# The cardiovascular actions of the isopropyl ester and other synthetic derivatives of palmitoyl carnitine

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Submitted by Katherine Ann Reeves for the degree of PhD of the University of Bath, 1995.







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## Summary

These studies have investigated the effects of some novel synthetic derivatives of palmitoyl carnitine in the isolated, perfused rat heart, isolated rat left atrium and isolated rat ventricular myocyte preparation.

In structure-activity relationship studies, it was found that the absence of a negatively charged group from palmitoyl carnitine, ie. the carboxylic acid group, produces compounds which are potent coronary dilators in the isolated perfused rat heart. It was also found that the positively charged quaternary nitrogen group was a necessary requirement for vasodilator activity.

Further studies were concerned with investigating the mechanism of action of one such coronary dilator derivative of palmitoyl carnitine, namely the isopropyl ester of palmitoyl carnitine (P1Pi), in the isolated perfused rat heart. It was found that this derivative was able to inhibit the coronary constrictor response to BAY K 8644, but not to caffeine or a low sodium perfusate. Additionally, P1Pi was able to inhibit the positive inotropic response to noradrenaline, prostaglandin $F_{2\alpha}$ and methoxamine, but not in response to a low sodium perfusate. The positive chronotropic response to noradrenaline was also unaffected.

In the isolated left atrium of the rat, P1Pi had no effect on noradrenaline-induced positive inotropic responses. However, in isolated rat ventricular myocytes, P1Pi was able to inhibit the noradrenaline-induced increase in the amplitude of calcium transients produced in response to electrical stimulation, measured with the fluorescent calcium probe fura-2.

In conclusion, P1Pi appears to be able to modulate both calcium entry through L-type calcium channels in smooth muscle and processes involving agonist-induced phosphorylation within the myocardium, but does not affect calcium release from the sarcoplasmic reticulum, sodium-calcium exchange or the pacemaker current $I_f$. It is proposed that these effects of P1Pi are mediated via an alteration of charge on the sarcolemmal surface of the cell.

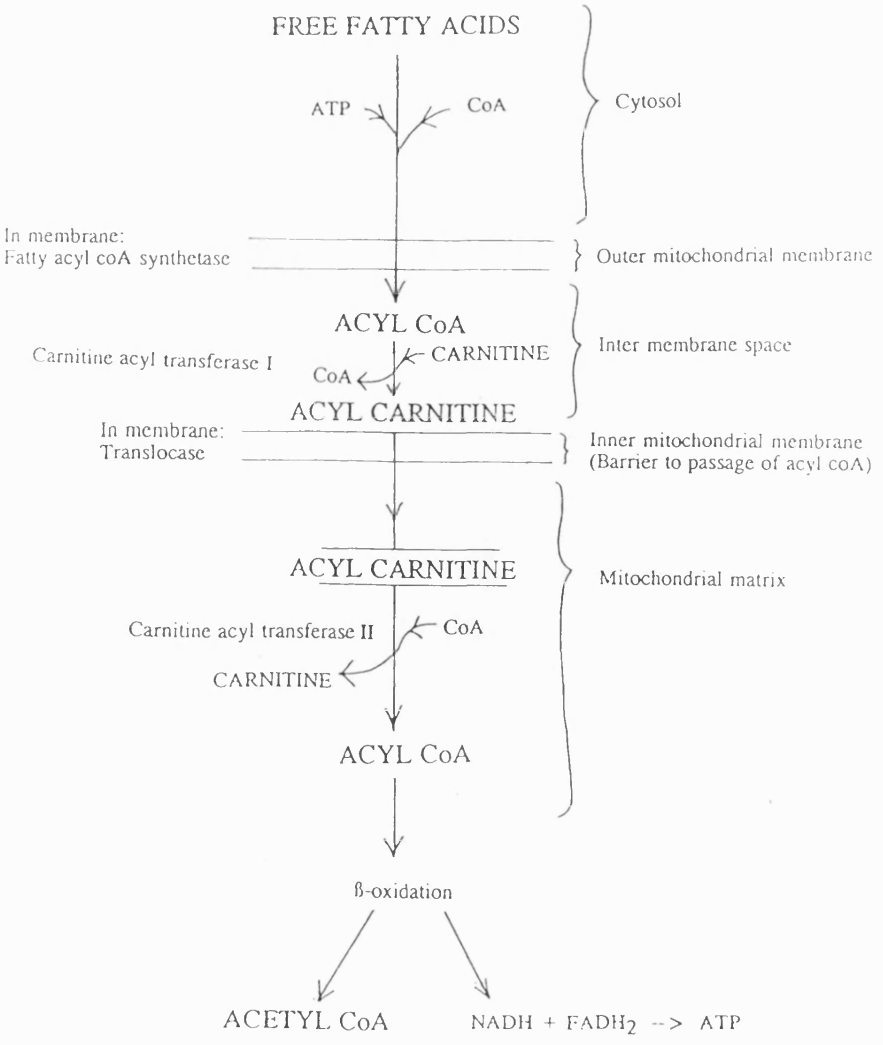
## Introduction

### 1. Role of acyl carnitines in fatty acid metabolism.

Acyl carnitines are compounds formed during the process of fatty acid metabolism within cells. In cardiac muscle, the main source of energy is from fatty acids which are transported across mitochondrial membranes for subsequent ß oxidation via the carrier molecule carnitine, which combines with fatty acids to form acyl carnitines (Fritz, 1963; Bremer, 1983). The scheme for this metabolic pathway is outlined below (Figure 1):-

**Figure 1**

The role of palmitoyl carnitine in fatty acid metabolism within the cell.



**2. Effects of acyl carnitines in the heart following accumulation under conditions of ischaemia.**

Under normal conditions carnitine is recycled following the dissociation of fatty acyl groups, catalysed by carnitine acyl transferase II (Fritz, 1963; Woeltje et al., 1990) and fatty acids are released within the mitochondrial matrix for subsequent ß oxidation. However, in the ischaemic myocardium there is insufficient oxygen present for ß oxidation of fatty acids and therefore there is a build up of both acyl CoA and acyl carnitine (Liedtke et al., 1978, Idell-Wenger et al., 1978; Katz & Messineo, 1981). Long chain acyl carnitines, a major constituent of which is palmitoyl carnitine, have been reported to increase 3.5 fold within 2 minutes of ischaemia in vivo (Datorre et al., 1991) and there is evidence to suggest that there is a preferential partitioning into the sarcolemma by way of their fatty acid chains (Fink & Gross, 1984; Meszaros, 1991). For example, Wu et al. (1993) measured a 100 fold increase in acyl carnitines in the junctional sarcolemma of isolated myocytes subjected to 10 minutes hypoxia, and Knabb et al. (1986) measured more than a 70 fold increase in the sarcolemma. This is likely to contribute to the membrane dysfunction and electrophysiological disturbances produced following ischaemia (Corr et al., 1984; Knabb et al., 1986). Heathers et al. (1987) reported an increase in $\alpha_1$-adrenoceptors in canine myocytes subject to hypoxia, correlating with an increase in endogenous long chain acyl carnitines. This has also been shown to occur under ischaemic conditions *in vivo* (Allely & Brown, 1988), and may contribute to the arrhythmogenic effect of acyl carnitines, as in separate studies α-adrenoceptor stimulation has been shown to be arrhythmogenic (Culling et al., 1987).

Accumulation of long chain acyl carnitines in the sarcolemma has been reported to produce cell-cell uncoupling due to a decrease in the gap junctional conductance (Knabb et al., 1986; Yamada et al., 1994; Wu et al., 1993), which has also been shown to contribute to the development of arrhythmias through re-entry pathways (Janse & Wit, 1989). Acyl carnitines have also been shown to produce an increase in

intracellular calcium within myocytes (Wu & Corr, 1992), which may be responsible for the calcium overload produced following ischaemia. The mechanism for the calcium increase has not yet been established. Spedding & Mir (1987) and Patmore et al. (1989) suggested that palmitoyl carnitine produced during ischaemia may act as an endogenous voltage gated calcium channel activator resembling BAY K 8644. However Wu & Corr (1992), directly measuring calcium currents in isolated myocytes found palmitoyl carnitine to actually inhibit the voltage dependent calcium current. They also found that palmitoyl carnitine delayed the inactivation of sodium inward current (Wu & Corr, 1994), and suggested that the increase in intracellular calcium produced in response to palmitoyl carnitine was a secondary effect due to an increase in $Ca^{2+}$ influx via $Na^{+}$-$Ca^{2+}$ exchange following a rise in intracellular $Na^{+}$, and subsequent calcium-induced calcium release from the sarcoplasmic reticulum. Palmitoyl carnitine has also been shown to directly activate the calcium release channel (ryanodine receptor) of the sarcoplasmic reticulum (El-Hayek et al., 1993), and if this effect occurs in myocytes, this may also contribute to the rise in intracellular calcium produced by palmitoyl carnitine. A direct inhibitory effect on $Na^{+}$ dependent $Ca^{2+}$ uptake via electrogenic $Na^{+}$-$Ca^{2+}$ exchange (Philipson & Nishimoto, 1982; Lamers et al., 1984) has also been reported for palmitoyl carnitine, which contradicts the mechanism of calcium increase proposed by Wu & Corr (1992; 1994) for palmitoyl carnitine, see above. However, far higher concentrations of palmitoyl carnitine were required for inhibition of $Na^{+}$-$Ca^{2+}$ exchange (up to 100$\mu$M for complete inhibition of the exchange process (Lamers et al., 1984)), than were used in Wu & Corr's studies (5-10$\mu$M). Palmitoyl carnitine has also been demonstrated to have an biphasic effect on protein kinase C activity, stimulatory at low concentrations and inhibitory at higher concentrations (Wise et al., 1982). Reports of the effects of palmitoyl carnitine on potassium currents and action potential duration have been equivocal. Some studies have shown an increase in action potential duration in response to lower concentrations of palmitoyl carnitine (Meszaros & Pappano, 1990), which may be related to the increase in membrane stabilisation and hence decrease in potassium permeability (Meszaros, 1991).

Conversely, others have shown a shortening of the action potential duration in response to palmitoyl carnitine (Corr, 1981; Wu & Corr, 1992), which may contribute to the arrhythmogenic action which occurs as a consequence of a reduction in action potential duration under ischaemic conditions. These discrepancies may be due to the fact that the effect on action potential duration appears to be dependent on the concentration of palmitoyl carnitine used, as at higher concentrations, Meszaros & Pappano (1990) also showed a decrease in action potential duration; additionally the effects of palmitoyl carnitine on other ion currents eg. current through L-type calcium channels must be taken into account in the effect on action potential, and the decrease in calcium current may contribute to the decrease in action potential duration shown by Wu & Corr (1992). Palmitoyl carnitine has also been shown to depolarise the resting membrane potential (Corr et al., 1984: Meszaros & Pappano, 1990), which may be explained by the inhibition of sodium current inactivation (Wu & Corr 1992; 1994), although modulation of the inward rectifier potassium channel current has also been proposed for this effect (Sato et al., 1993). The depolarisation of resting membrane potential by palmitoyl carnitine may also contribute to increased automaticity and the development of arrhythmias.

The actions of palmitoyl carnitine at concentrations up to 1$\mu$M may be related to their ability to stabilise cell membranes (Meszaros, 1991; Epand & Lester, 1990). At higher concentrations, $>30\mu$M, palmitoyl carnitine has been shown to produce myocyte disruption (Meszaros & Pappano, 1990), through its amphiphilic detergent action (Corr et al., 1984) and this may account for the multiple effects on membrane bound enzymes at higher concentrations for example, inhibition of $Na^+$-$K^+$-ATPase (Adams et al., 1979), inhibition of $Ca^{2+}$-ATPase in the sarcoplasmic reticulum (Pitts et al., 1978).

The structure of palmitoyl carnitine is shown in Figure 2 (see below). Like all long chain acyl carnitines, palmitoyl carnitine is an amphiphilic zwitterion containing

lipophobic positively (quaternary nitrogen) and negatively (carboxylic acid) charged groups, as well as a lipophilic fatty acid chain (palmitoyl).

### 3. Effects of palmitoyl carnitine on smooth muscle.

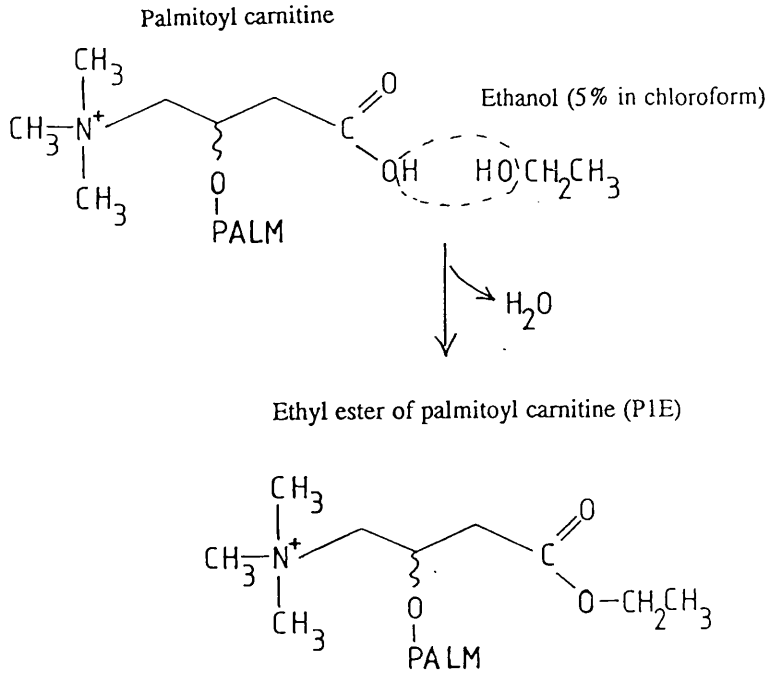
Fewer studies have been carried out to investigate the effects of acyl carnitines in smooth muscle. However, Corr et al. (1984) reported that palmitoyl carnitine is able to leak into the extracellular space during prolonged ischaemia, and therefore any vascular effects of acyl carnitines may be of relevance to ischaemic injury. Spedding and Mir (1987) found that in potassium depolarised smooth muscle of guinea pig taenia coli, palmitoyl carnitine produced a contraction resembling the L-type calcium channel agonist BAY K 8644, and the same effect has been described in contracting depolarised rat aorta (Dainty et al., 1990). Palmitoyl carnitine has also been reported to inhibit both basal and receptor mediated release of EDRF from the endothelium (Dainty et al., 1990; Inoue et al., 1994).

### 4. Effects of exogenous palmitoyl carnitine in the isolated perfused rat heart.

In terms of coronary vascular effects, Criddle et al. (1987; 1990) demonstrated that palmitoyl carnitine can produce a coronary constrictor effect in the isolated perfused rat heart, together with an irreversible depressor action on cardiac contractility and no effect on heart rate. Whilst investigating the vascular effects of palmitoyl carnitine in the isolated whole heart, Criddle et al. (1987; 1988) observed an anomalous response, namely a coronary dilation, produced in response to palmitoyl carnitine in some preparations. Further investigation led to the discovery that palmitoyl carnitine had become contaminated with the ethyl ester derivative, formed from the ethanol contained within the storage medium (Criddle et al., 1990). The reaction is outlined below (Figure 2).

**Figure 2**

Reaction for the esterification of palmitoyl carnitine.




**5. Actions of novel ester derivatives of palmitoyl carnitine in isolated perfused rat heart.**

Upon purification, this ethyl ester derivative, as well as other ester derivatives of palmitoyl carnitine, were found to be pure dilators in the isolated perfused rat heart. At the doses used, no effect on either basal contractility or basal heart rate was produced. It can be seen from the above figure that the esterification of palmitoyl carnitine converts the molecule from a zwitterion with an overall neutral charge to a molecule with a single positive charge. Initial structure-activity relationship studies showed that the dilator activity of these novel acyl carnitine derivatives is highly dependent on the length of the fatty acid (acyl) chain (Criddle et al., 1991). When acyl chain length vs. coronary dilator potency for the ethyl ester derivatives of acyl carnitines was plotted, a bell shaped curve was obtained, with a 16 carbon fatty acid chain length, palmitoyl, producing the greatest coronary dilator potency. Altering the ester grouping at the C terminus of the carnitine moiety had a less profound effect on

dilator activity (Criddle et al., 1990). However, the isopropyl ester of palmitoyl carnitine (P1Pi) was found to be marginally more potent than the ethyl ester first discovered. Therefore, P1Pi was selected as the reference dilator compound and used in subsequent experiments investigating the mechanism of action of such compounds.

**6. Action of the isopropyl ester of palmitoyl carnitine in other tissues.**

Further studies investigating the action of P1Pi have shown it to be a potent vasodilator in other perfused vascular beds including the mesenteric vascular bed, the perfused rat tail vascular bed (via caudal artery) and the isolated perfused rat kidney (Figure 3).

**Figure 3**

Actions of P1Pi in isolated vascular preparations from the rat; the isolated, perfused rat heart (a), mesenteric vascular bed (b), isolated perfused kidney (c) and perfused rat tail (d).

a. In the isolated, perfused rat heart, compared with the actions of amyl nitrate (AN), verapamil (VER), iloprost (ILO), ethyl ester of palmitoyl carnitine (P1E) and cromakalim (Crk). Effects on developed tension (DT), heart rate (HR) and perfusion pressure (PP).

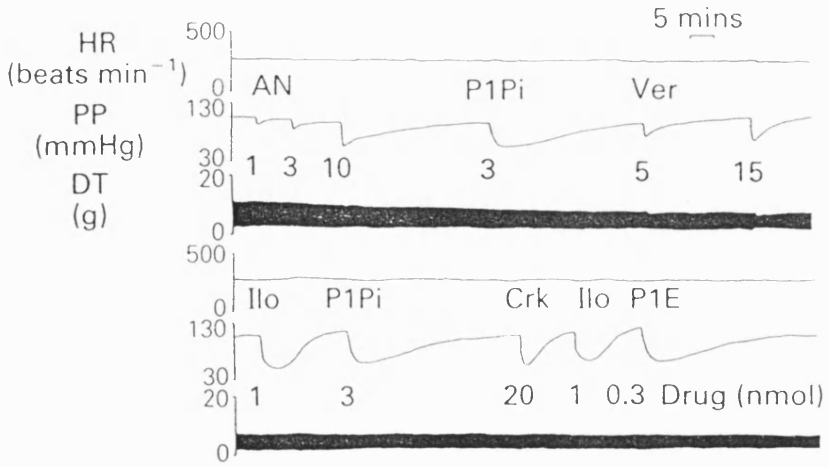
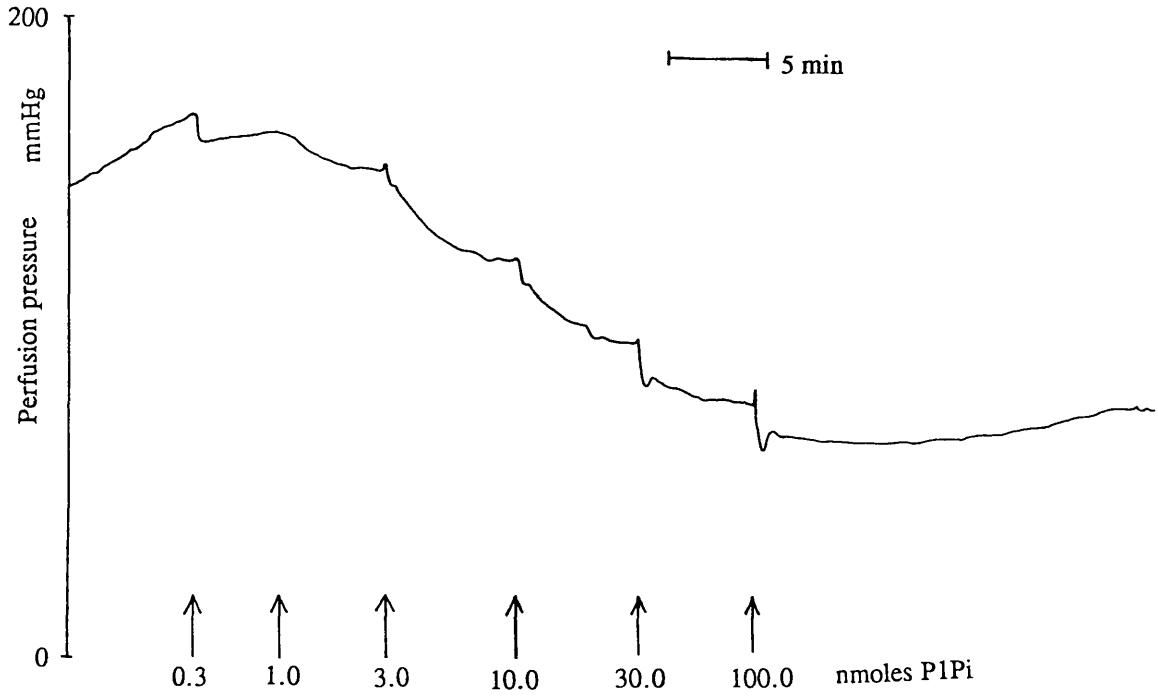
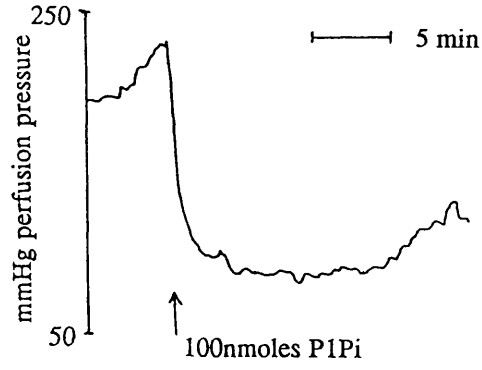


**Figure 3 cont.**

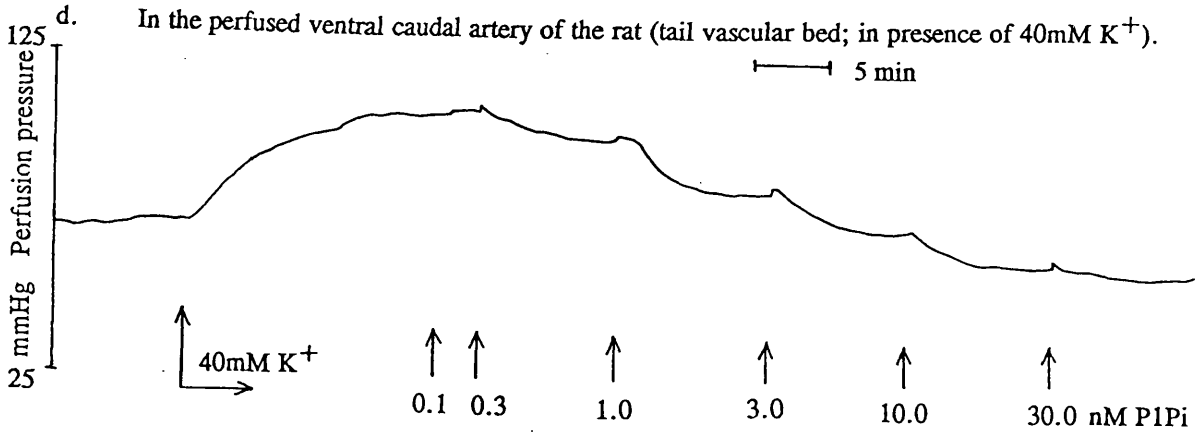
b. In isolated, perfused mesenteric vascular bed of rat (in presence of 110mM $K^+$).




c. In the isolated, perfused rat kidney (In the presence of 20mM $K^+$).




d. In the perfused ventral caudal artery of the rat (tail vascular bed; in presence of 40mM $K^+$).



In contrast to the effects in perfused vascular preparations, containing an intact microvasculature, P1Pi was found to be inactive in isolated smooth muscles and conduit vessels such as the guinea pig taenia coli and the rat hepatic portal vein or preconstricted rat aorta (Criddle et al., 1992).

**7. Mechanism of action of the isopropyl ester of palmitoyl carnitine (P1Pi).**

Studies investigating the mechanism of action of P1Pi have mainly been carried out using the isolated perfused rat heart and the mesenteric vascular bed of the rat. These studies have concluded that the vasodilator action of P1Pi is via a direct action on the smooth muscle rather than via the release of EDRF (NO), or EDHF, from endothelial cells (Criddle et al. 1994), as in air damaged mesenteric vascular beds, the vasodilator effect of P1Pi was not attenuated. In addition, in whole hearts vasodilator responses were not attenuated by L-$N^G$-nitroarginine, or enhanced by superoxide dismutase, also indicating a lack of involvement of EDRF (NO). Criddle et al. (1994) also concluded that the vasodilator action of P1Pi does not appear to be mediated via the release of arachidonic acid metabolites as the cyclo-oxygenase inhibitor flurbiprofen and the dual cyclo-oxygenase / 5-lipoxygenase inhibitor BW755C had no effect on the vasodilator response. The action is not mediated via activation of bradykinin $B_2$ receptors as the selective antagonist D-$ARG^0$[$HYP^3$-$THI^{5,8}$-D-$PHE^7$]bradykinin did not attenuate the response. Finally, there appears to be no involvement of ATP dependent $K^+$ channels in the dilator action of P1Pi, as responses were unaffected by the ATP dependent $K^+$ channel blocker glibenclamide. The fact that P1Pi also inhibited calcium-induced constrictions in the 110mM $K^+$ depolarised mesenteric vascular bed (Criddle et al., 1992; 1994) also implies lack of involvement of other types of $K^+$ channel. However, unpublished observations showed that the addition of negatively charged dextran to the perfusion medium of isolated rat hearts was found to attenuate the P1Pi-induced coronary dilator action, indicating that the positive charge on the molecule is likely to be important in eliciting

a vasodilator response. This could explain the different effects of P1Pi, compared with those of the parent compound palmitoyl carnitine, which is a zwitterion and therefore overall neutral molecule. It would also indicate a site of action on the outer sarcolemmal surface, possibly affecting ion channel function, as charged compounds cannot cross sarcolemmal membranes due to the hydrophobic core of the lipid bilayer (Katz, 1992). Other positively charged amphiphiles, for example polymyxin B (Burt et al., 1983) and dodecyltrimethylammonium (DDTMA) (Post et al.,1991), have been shown to decrease the calcium current through L-type calcium channels in myocardial cells, while negatively charged amphiphiles, for example dodecylsulphate (DDS), increase it (Post et al., 1991). The ability of positively charged amphiphiles to decrease calcium current is probably due a decrease in the anionic surface charge on the external sarcolemmal surface, produced by binding of the cationic amphiphile, which increases the potential difference across the sarcolemma, thus impairing the excitability of the voltage gated calcium channel (Green & Andersen,1991; Latorre et al., 1992). Although P1Pi had no effect on basal cardiac contractility, this may explain the action of the positively charged amphiphile on vascular smooth muscle. As Criddle et al. (1992; 1994) also showed that in the mesenteric vascular bed, P1Pi is able to attenuate the constrictor action of calcium ions in potassium depolarised preparations, this indicates that P1Pi is able to inhibit voltage gated calcium channels in smooth muscle.

Interestingly, the parent molecule palmitoyl carnitine has itself been shown to reduce surface negative charge within the myocardium (Inoue & Pappano,1983; Meszaros et al., 1988; Meszaros, 1991), which would indicate an inhibitory effect of palmitoyl carnitine, as well as the ester derivatives, on the L-type calcium channel, as well as other excitatory ion currents eg. sodium currents, and several studies (Inoue & Pappano, 1983; Wu & Corr, 1992; Sato et al., 1992) have shown that this is indeed the case, at least in myocytes. Other mechanisms are therefore likely to be responsible for the vasoconstrictor action of palmitoyl carnitine in coronary smooth muscle eg. via

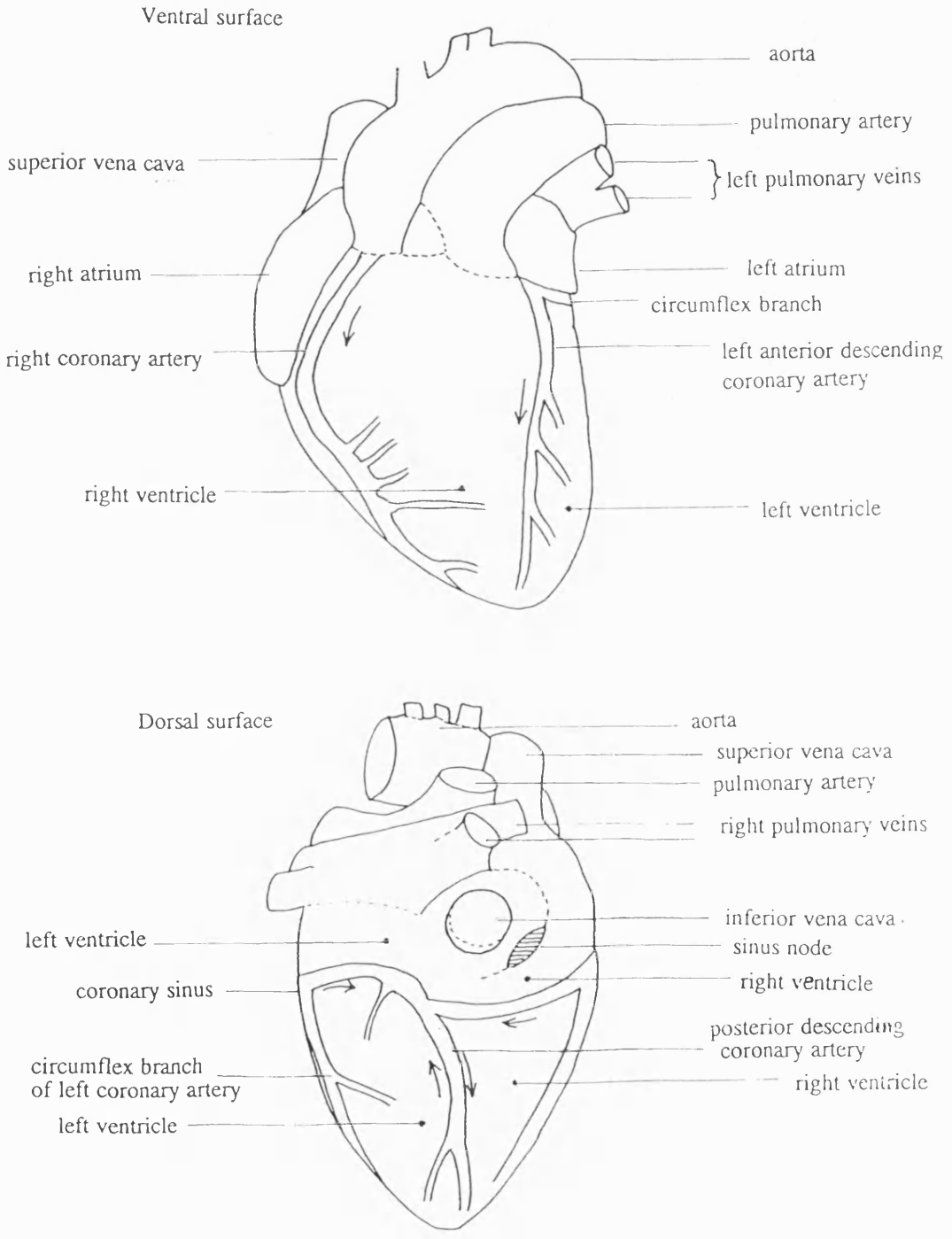
$Na^+$-$Ca^{2+}$ exchange, secondary to an increase in intracellular sodium or calcium release from the sarcoplasmic reticulum (See above).

**8. Aims of the present study.**

The initial studies described in this report were carried out in the isolated perfused rat heart, to investigate the effects of the novel acyl carnitine derivatives on the coronary vasculature (Figure 4). Firstly, to perform further structure-activity relationship studies on novel acyl carnitine derivatives, in order to pin-point the features of the molecule which confer vasodilator activity. By studying compounds containing different groupings in place of the isopropyl ester group at the C terminus of the molecule, it was possible to investigate whether an ester group is important in conferring coronary dilator activity. By keeping the C terminus constant and changing the groups attached to the quaternary nitrogen at the N terminus of the molecule, the importance of charge on activity was investigated. In addition, these studies further investigate the mechanisms of action of these novel vasodilator acyl carnitine derivatives, in particular P1Pi, in the isolated perfused rat heart preparation. This preparation allows the study of the action of agents on the coronary vasculature and the cardiac muscle simultaneously, by perfusion of the coronary vessels via retrograde perfusion of the aorta (Figure 4).

**Figure 4**

The isolated heart showing the main coronary vessels.



During the course of these experiments an additional property of P1Pi was discovered, namely the ability to suppress agonist-induced increases in coronary contractility whilst having no significant effect on basal contractility in isolated perfused heart preparations. Studies were therefore carried out using isolated atria and isolated myocyte preparations as well as whole hearts, in order to elucidate the mechanisms involved in this additional property of P1Pi. In order to investigate the mechanism of action of P1Pi in the coronary vasculature and the myocardium, the effect of P1Pi on responses to BAY K 8644, noradrenaline, methoxamine, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), caffeine and a low sodium perfusate were investigated, which are able to increase myocardial contractility and produce smooth muscle contraction in different ways, outlined below.

**8.1 Mechanisms of smooth muscle contraction.**

In vascular smooth muscle, BAY K 8644, a dihydropyridine calcium channel activator, is able to increase calcium entry into smooth muscle cells through L-type calcium channels (Schramm et al., 1983) which may directly activate the contractile filaments, or promote calcium-induced calcium release (Ito et al.,1991) which augments the increase in calcium within the smooth muscle cell. Caffeine directly activates the ryanodine-sensitive calcium release channel of the sarcoplasmic reticulum (SR), to promote calcium release within the smooth muscle cell independent of calcium entry, (Van Breemen & Saida, 1989; Watanabe et al., 1992). However, it can also promote relaxation in cells containing basal cAMP or cGMP levels, through an increase in intracellular cAMP and cGMP, via inhibition of phosphodiesterase (Butcher & Sutherland, 1962), to increase calcium uptake by the sarcoplasmic reticulum and enhance efflux via sarcolemmal $Ca^{2+}$-ATPase (Van Breemen & Saida, 1989). An increase in intracellular cAMP in smooth muscle may also promote relaxation via a decrease in the sensitivity of the contractile proteins in smooth muscle (Kerrick & Hoar, 1981). In vascular smooth muscle, noradrenaline and methoxamine

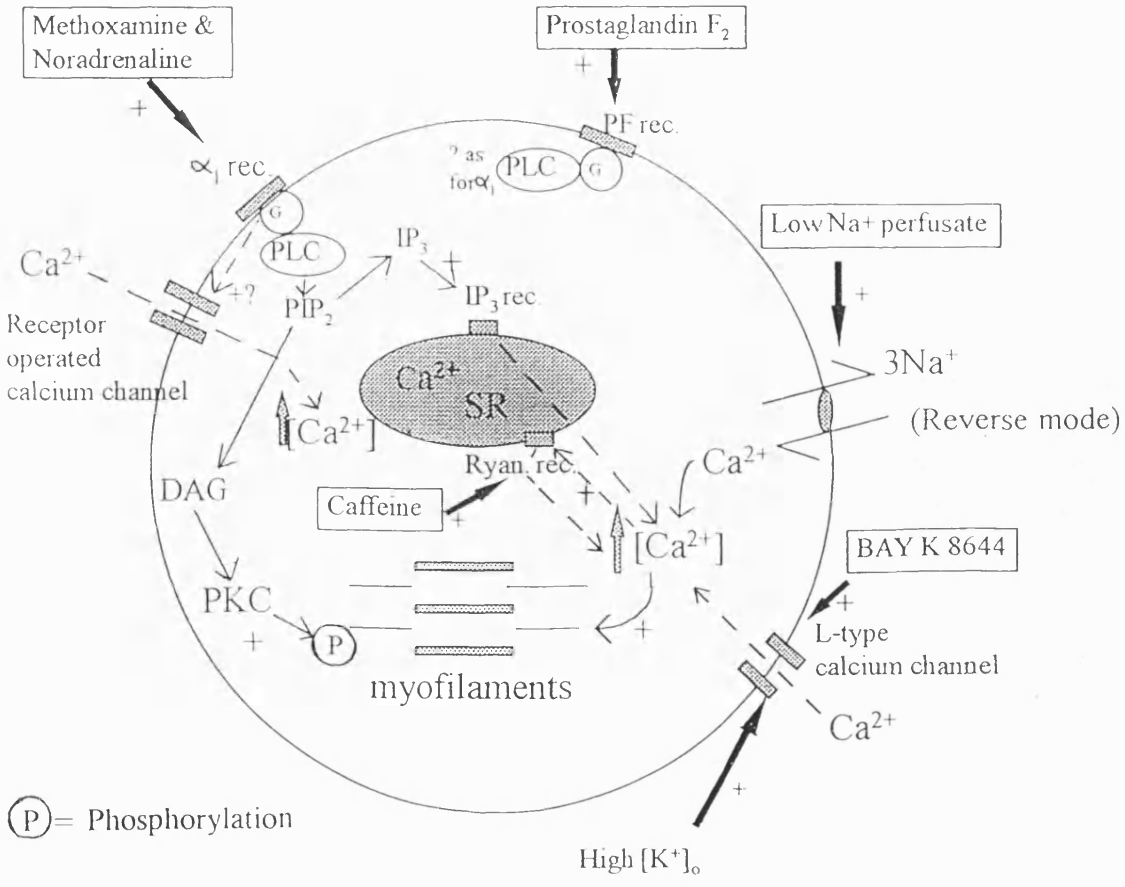
can activate $\alpha_1$-adrenoceptors which leads to activation of phospholipase C (PLC) via a G-protein, to promote hydrolysis of phosphatidyl inositol (4,5) bisphosphate ($PIP_2$), to form inositol (1,4,5) triphosphate ($IP_3$) and diacyl glycerol (DAG). This produces both an increase in calcium release from the sarcoplasmic reticulum, via the action of $IP_3$ on the calcium release channel ($IP_3$ receptor of the sarcoplasmic reticulum) and a phosphorylationof the contractile proteins following DAG activation of protein kinase C (PKC) (Nishimura et al., 1989; Van Breemen & Saida, 1989; Morgan & Suematsu, 1990). Stimulation of $\alpha_1$-adrenoceptors may also promote calcium entry, through receptor operated (voltage-insensitive) calcium channels (Van Breemen & Saida, 1989; Lee & Severson, 1994). $PGF_{2\alpha}$-induced vasoconstriction has been reported to involve a similar mechanism, ie. via activation of phospholipase C (Hatanaka et al., 1989; Coleman et al., 1994), following activation of prostaglandin FP receptors, although thromboxane receptors could also be involved in $PGF_{2\alpha}$-induced vasoconstriction (Schrör, 1993). Agonist-induced calcium entry is generally regarded as a more effective mechanism for increasing intracellular calcium in smooth muscle cells than depolarisation or direct activation of calcium channels, through an inhibition of the 'buffer barrier'. The buffer barrier describes a calcium buffering process within smooth muscle cells, whereby calcium entering the cell is taken up by the superficial sarcoplasmic reticulum, to be extruded via the sarcolemmal $Ca^{2+}$-ATPase pump, and $Na^{+}$-$Ca^{2+}$ exchange. As agonists can increase calcium release as well as calcium entry, they are more effective in increasing calcium levels in the bulk of the cytoplasm in smooth muscle cells (Van Breemen & Saida, 1989; Nishimura et al., 1989; Sturek et al., 1992). A low sodium perfusate can also promote an increase in calcium in vascular smooth muscle cells, producing vasoconstriction. A low sodium concentration outside the smooth muscle cell will favour calcium influx and sodium efflux via the exchanger (Batlle et al., 1991), and hence promote calcium-induced calcium release from the sarcoplasmic reticulum (Ito et al., 1981), to provide the calcium for activation of myofilaments, and hence promote contraction. A high potassium perfusate depolarises the cell which opens calcium channels to promote calcium entry. Hence, high potassium facilitates the action of calcium antagonists, but

above a certain threshold (approximately 40mM) of potassium, will prevent the action of vasodilators which act by opening potassium channels, due to a high degree of depolarisation of the smooth muscle cells and a reduction in the driving force for potassium efflux following potassium channel opening (Hamilton et al., 1986).

In smooth muscle, the main trigger for contraction following a rise in intracellular calcium is by calcium binding to calmodulin, to activate myosin light chain kinase and trigger actin mediated hydrolysis of myosin Mg ATPase and cross-bridge cycling. However, in smooth muscle, contraction can occur in the absence of an increase in intracellular calcium, for example via PKC-induced phosphorylation of myosin light chain, which contributes to the $\alpha_1$-adrenenoceptor mediated vasoconstriction (Walsh, 1990; Morgan & Suematsu, 1990). The pathways involved in smooth muscle responses to the agents used in this study are outlined in Figure 5a.

**Figure 5a**

**Different mechanisms of contraction within smooth muscle cells, elicited by the compounds used in this study.**



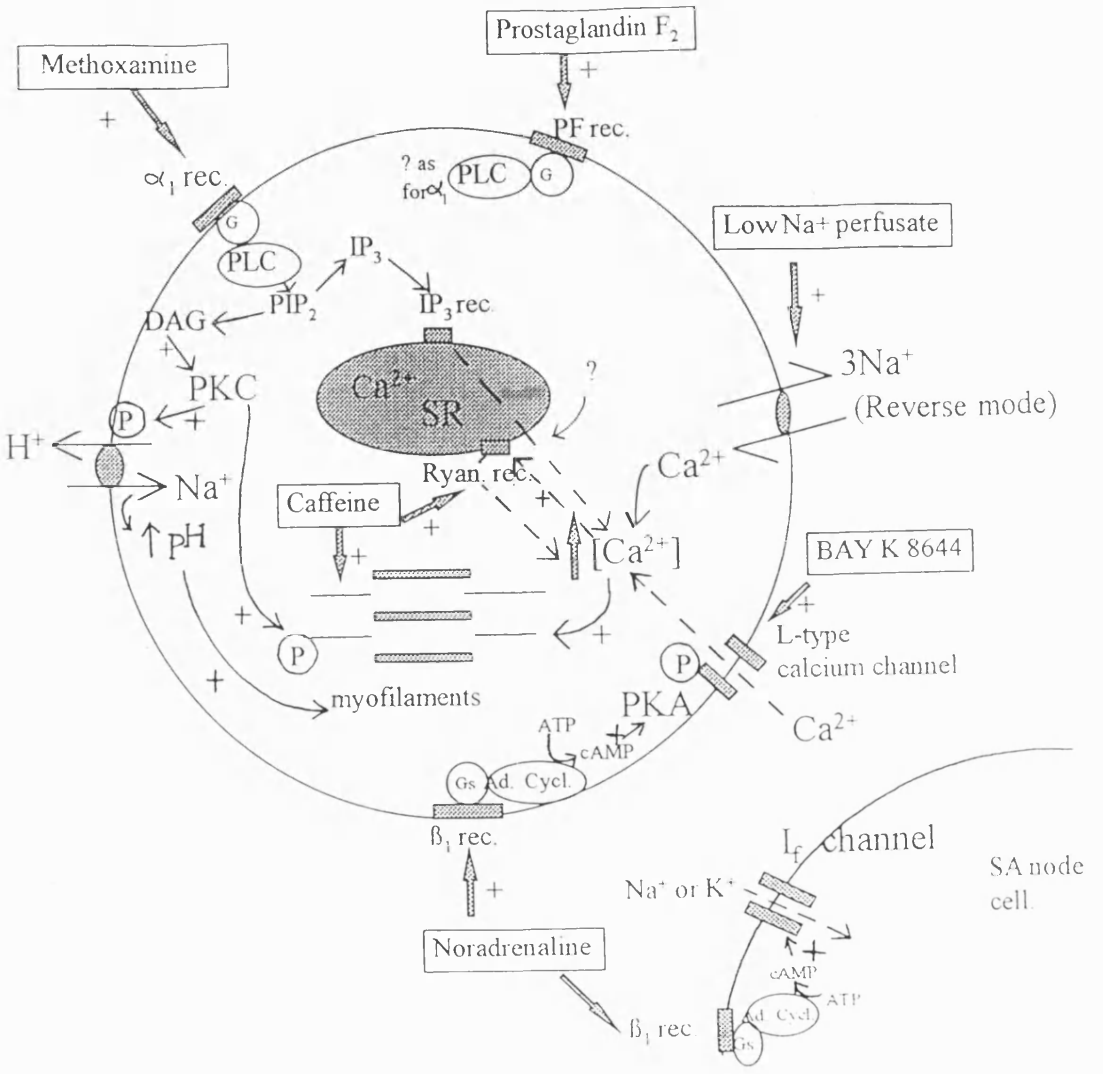
## 8.2 Mechanisms of increasing cardiac muscle contractility.

In the myocardium, the same agents can increase contractility in the following ways. The calcium channel activator BAY K 8644 can increase calcium entry through L-type calcium channels in cardiac muscle (Thomas et al., 1985) and hence promote an increase in calcium-induced calcium release from the sarcoplasmic reticulum (Fabiato, 1983; Rich et al., 1988). Noradrenaline can increase force of contraction by similarly increasing calcium current through L-type calcium channels. In this case, this follows $\beta_1$-adrenoceptor stimulated $G_S$-protein activation of adenylate cyclase and a subsequent increase in intracellular cAMP. This activates protein kinase A, which phosphorylates L-type calcium channels to increase the probability of opening (Sperelakis, 1988; Robishaw & Foster, 1989). Protein kinase A can also increase the rate of relaxation of cardiac muscle both via increasing the rate of uptake of calcium into the sarcoplasmic reticulum via phosphorylation of phospholamban, which removes the inhibitory action of phospholamban on the sarcoplasmic reticulum $Ca^+$-ATPase pump (Kranias, 1985), and also via phosphorylation of the myofilaments to increase cycling rate (Strang et al., 1994). In the whole heart, the increase in cAMP is also able to activate the pacemaker current $I_f$, carried by sodium and potassium ions in sino-atrial node cells (Difrancesco, 1993), and hence increase heart rate, mainly via a direct effect of cAMP rather than via protein kinase A-induced phosphorylation (Difrancesco & Tortora, 1991; Difrancesco & Mangoni, 1994). A direct $G_S$ protein pathway for the activation of $I_f$ current, following ß-adrenoceptor stimulation has also been proposed (Yatani et al., 1990). The mechanism by which methoxamine, via $\alpha_1$ -adrenoceptor stimulation, promotes an increase in force of contraction of cardiac muscle has not yet been fully established, but is thought to be independent of a rise in intracellular calcium (Puceat et al., 1992). The $\alpha_1$-adrenoceptor mediated $PIP_2$ hydrolysis, as described above for smooth muscle, results in formation of $IP_3$ and DAG (Brown et al., 1985). However, $IP_3$ is not thought to have an important role in myocardial contractility (Woodcock et al., 1987; Langer, 1992). DAG-activated protein kinase C, however,

is thought to increase myocardial contractility, both via direct phosphorylation of contractile proteins to increase the sensitivity to calcium (Puceat et al., 1990), and via intracellular alkalisation by phosphorylation of the sarcolemmal $Na^+$-$H^+$ exchanger to promote $H^+$ efflux (Gambassi et al., 1992; Fedida et al., 1993). Prostaglandin $F_{2\alpha}$, which has also been shown to increase myocardial contractility (Karmazyn et al., 1981) may also produce a similar effect, as the effector mechanism is via phospholipase C, and $PIP_2$ hydrolysis (Hatanaka et al., 1989; Coleman et al., 1994). However, $PGF_{2\alpha}$ has also been reported to inhibit $Na^+$-$K^+$ATPase (Karmazyn et al., 1981), and thus may produce an increase in contractility via a similar mechanism to the cardiac glycosides, via increased calcium entry through reverse $Na^+$-$Ca^{2+}$ exchange, and subsequent release of calcium from the sarcoplasmic reticulum (Kohmoto et al., 1994). A low sodium perfusate can increase myocardial contractility in a similar manner to the mode of action described for the smooth muscle contraction (see above), ie. an increase in calcium influx via the exchanger and calcium-induced calcium release from the sarcoplasmic reticulum (Leblanc & Hume, 1990, Kohmoto et al., 1994). Caffeine can produce a direct activation of the ryanodine sensitive calcium release channel (Rousseau & Meissner, 1989), as well as an increase in myofilament responsiveness to calcium, and an increase in calcium entry following phosphorylation of L-type calcium channels following an increase in intracellular cAMP, through an inhibition of phosphodiesterase (Bers, 1985), assuming basal levels of cAMP to be present.
The main trigger for contraction in cardiac muscle, following a rise in intracellular calcium, is calcium binding to troponin C, which shifts troponin I and tropomyosin relative to actin to permit actin to cross-bridge myosin, with the hydrolysis of myosin MgATPase and the production of force (Langer, 1992). Unlike smooth muscle, force is only produced in the presence of an increase in intracellular calcium, although phosphorylation of contractile proteins eg. by PKC can increase the sensitivity of the myofilaments to calcium, and hence increase force of contraction. The mechanisms by which the agents used in this study can increase myocardial contractility are outlined in Figure 5b.

Figure 5b.

**The different mechanisms of increasing myocardial contractility, elicited by the compounds used in this study.**




This study investigates the interaction of the coronary dilator P1Pi with the above agents in isolated perfused hearts, isolated left atria and isolated myocytes, in order to elucidate the mechanisms of action of P1Pi within coronary smooth muscle and cardiac muscle. It also investigates structure-activity relationships of coronary dilator acyl carnitine derivatives using the isolated, perfused rat heart model, as discussed above. In addition, the effects of the coronary dilator P1Pi on a model of ischaemia / reperfusion, using the isolated, perfused rat heart were studied, in order to investigate a possible protective role.

## Methods

### 1. Isolated perfused rat heart (Langendorff) technique.

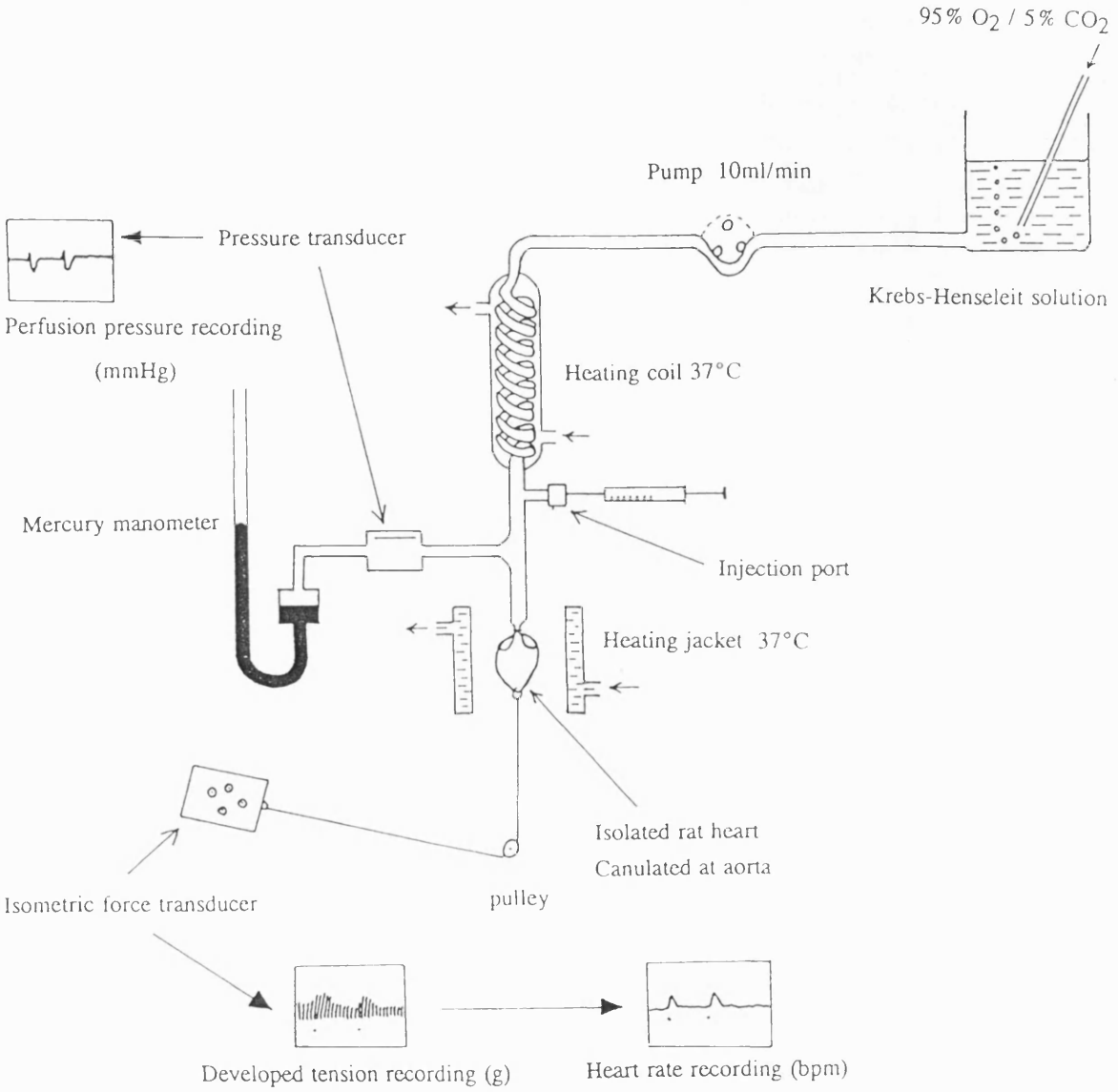
Male Wistar rats (270-320g) were anaesthetised with sodium pentobarbitone (60mg/kg i.p.) and the hearts excised and perfused at 10ml/min, 37°C using a modified Langendorff technique. The Krebs-Henseleit solution used was of the following composition (mM):-

| | | |
|---|---|---|
| NaCl: | 118 | |
| $NaHCO_3$: | 25 | |
| KCl: | 4.7 | Gassed with 95% $O_2$ 5% $CO_2$ |
| $KH_2PO_4$: | 1.2 | pH 7.4 |
| $MgSO_4$: | 1.2 | |
| L-Glucose: | 11.1 | |
| $CaCl_2$: | 2.4 | |

Hearts were perfused via the coronary vessels, following retrograde perfusion of the aorta. The experimental setup for this technique is shown in Figure 6.

**Figure 6**

The isolated perfused rat heart (Langendorff) technique, for the measurement of coronary perfusion pressure, developed tension and heart rate.



In preparations in which coronary dilator responses were measured, after 20 minutes of perfusion the perfusate was switched to one containing 2mM KCl ie. the total $K^+$ concentration was reduced from 5.9mM to 3.2mM. This had the effect of raising the coronary perfusion pressure by an average of 27mmHg; from 70±2.9mmHg to 97±2.6mmHg, n=69, to allow greater scope for the coronary dilator responses. Coronary perfusion pressure was measured using a Gould pressure transducer attached to a side arm on the aortic cannula, developed tension was recorded under a resting tension of 2g via a hook placed in the apex of the heart, connected to a Devices isometric transducer. The tension recording triggered a Gould rate meter to give a concurrent heart rate record. All recordings were made on Gould 3400/2400 recorders and drugs were added either as perfusions or in volumes of no more than 30$\mu$l via an injection port on the aortic cannula.

**1.1 Structure-activity relationship studies on novel synthetic acyl carnitine derivatives.**

Following a 20 minute equilibration period, the $K^+$ concentration of the Krebs-Henseleit solution was switched to 3.2mM. The potencies of several structural derivatives of palmitoyl carnitine were compared with the prototype coronary dilator derivative, the isopropyl ester of palmitoyl carnitine (P1Pi). Therefore a submaximal dose (0.3nmoles) of P1Pi was first administered to the preparation. The preparation was allowed to recover before constructing a dose-response curve to the compound under investigation, allowing the preparation to recover between each addition.

**1.2 Effect of verapamil on the response to Compound G.**

Hearts were set up as described above, and the same protocol repeated using one of the synthetic derivatives of palmitoyl carnitine, compound G (table 1, results), as the

compound under investigation. However, in these experiments 100nM verapamil was perfused prior to the addition of the 0.3nmole P1Pi standard and was present for the duration of the experiment.

**1.3 Effect of 110mM $K^+$ perfusion on responses to P1Pi, verapamil, papaverine, salbutamol, nicardipine and lemakalim, and the effect of 20mM $K^+$ perfusion on responses to P1Pi and verapamil.**

Following equilibration in 3.2mM $K^+$ Krebs-Henseleit, a bolus dose of 0.3nmoles P1Pi was administered, and following recovery, a bolus sub-maximal dose of another coronary dilator was administered (verapamil 1nmole, papaverine 30nmoles, salbutamol 50nmoles, lemakalim 10nmoles or nicardipine 0.5nmoles). This process was repeated before perfusing a modified Krebs-Henseleit solution, in which the potassium chloride concentration had been increased to 108.8mM ie. 110mM $K^+$ in total. The sodium chloride concentration was reduced to 13.9mM to maintain osmolarity. The same doses of P1Pi and the other coronary dilator used were then repeated in the presence of 110mM $K^+$. In the case of 20mM $K^+$ experiments, the same protocol was repeated using a modified Krebs-Henseleit containing 18.8mM KCl, 103.9mM NaCl ie. 20mM $K^+$, in place of 110mM $K^+$. In these experiments, the effect on P1Pi and verapamil responses only were examined.

**1.4 Effect of tetraethylammonium chloride (TEA) on responses to P1Pi.**

Hearts were equilibrated in 3.2mM $K^+$ and single doses of verapamil (1nmole) and P1Pi (1nmole), as control responses, were administered before a concentration of 10mM TEA was perfused through the preparation for 20 minutes. A bolus dose of 1nmole verapamil was repeated in the presence of the TEA before constructing a dose-response curve to P1Pi in the presence of TEA.

**1.5 Effect of P1Pi, palmitoyl carnitine, atenolol or polymyxin B on the developed tension, heart rate and perfusion pressure responses to BAY K 8644, noradrenaline, $PGF_{2\alpha}$, methoxamine or caffeine.**

These experiments were conducted in the presence of a 5.9mM $K^+$ perfusate throughout. Following 20 minutes equilibration a dose-response curve to bolus doses of BAY K 8644, noradrenaline, $PGF_{2\alpha}$, methoxamine or caffeine was constructed, allowing the preparation to recover between each dose. At the end of the initial dose-response curve, a concentration of P1Pi, palmitoyl carnitine, phentolamine, atenolol or polymyxin B was perfused for 20 minutes before repeating the dose-response curve in the presence of the perfused compound.

In the case of BAY K 8644 experiments, further dose-response curves were constructed at 20 minute intervals in the presence of increasing concentrations of P1Pi (10nM to 1$\mu$M). In all cases, time-matched dose-response curves were repeated in the absence of any compound being perfused. In the case of the methoxamine experiments, only one dose-response curve was constructed in each experiment, either in the presence or absence of P1Pi, due to the tachyphylaxis shown with this compound.

**1.6 Effect of noradrenaline following washout of P1Pi (reversibility experiments).**

In experiments to investigate the reversibility of the action of P1Pi on the noradrenaline-induced responses, hearts were perfused with 5.9mm $K^+$ Krebs-Henseleit and following 20 minutes equilibration, a single submaximal dose of noradrenaline (1nmole) was added. This dose was repeated after perfusing P1Pi through the preparation for 20 minutes. The P1Pi was washed out for 40 minutes before applying the same dose of noradrenaline for a third time.

**1.7 Effect of increasing heart rate by electrical pacing in the isolated perfused heart on developed tension and perfusion pressure.**

Hearts were set up in 5.9mM $K^+$ Krebs-Henseleit solution, and allowed 20 minutes to equilibrate. An electrode was placed on the right atrium and another on the aortic cannula, connected to a Grass SD9 stimulator. A threshold voltage was established and the voltage was then set 50% above this, pulse width 1ms. Hearts were stimulated first at 330bpm for 30 seconds, and allowed to recover. Heart rate was subsequently increased to 360, 390, 420, 450bpm allowing the preparation to recover between each period of stimulation.

**1.8 Effect of lowering the concentration of sodium chloride in the Krebs-Henseleit perfusate on developed tension, heart rate and perfusion pressure, and the effect of P1Pi on the response to a low (59mM) concentration of NaCl in perfusate.**

Hearts were set up as previously described. In order to establish a concentration of sodium chloride producing a submaximal response in terms of myocardial contractility, a concentration-effect curve to the sodium chloride concentration of the Krebs-Henseleit perfusate, starting at a baseline of 118mM (normal Krebs) and being reduced to 89, 59 & 44mM respectively at five minute intervals, was constructed. Isotonicity was maintained by the addition of 0, 59, 118 & 148mM sucrose respectively.

Further experiments were subsequently carried out using 59mM NaCl, isotonicity being maintained by the addition of sucrose (118mM). In these experiments, the low sodium perfusate was perfused for 5 minutes before returning to the normal perfusate. This was repeated three times at 20 minute intervals. In the control experiments it was found that the second and third application of the low sodium perfusate produced

responses which were not significantly different from each other. Therefore in test experiments P1Pi was perfused for 20 minutes between the second and third low sodium periods, comparisons being made with the second and third low sodium periods in time-matched controls.

**1.9 Effect of P1Pi or palmitoyl carnitine on the effects of 3x 20 minutes zero flow, global ischaemia / reperfusion in the isolated heart.**

Hearts were set up in 5.9mM $K^+$ modified Krebs-Henseleit, with 11mM glucose in Krebs replaced by 2mM. This concentration of glucose was selected as it had been previously been shown by our laboratory to be the threshold concentration for ATP depletion and ischaemic contracture in a model of 20 minutes ischaemia / 20 minutes reperfusion, and thus a graded effect on the effects of each 20 minute period of ischaemia and reperfusion could be obtained. Following a 20 minute period of equilibration, hearts were subjected to 20 minutes zero flow global ischaemia. The preparation was kept warm by means of a heating jacket maintained at 37°C. Following this period flow was resumed, and after 20 minutes of reperfusion the whole process was repeated. The whole process was repeated for a third time. In separate experiments, a concentration of P1Pi or palmitoyl carnitine was included in the perfusate throughout.

**2. The isolated stimulated left atrium of the rat.**

Male Wistar rats (270-320g) were anaesthetised with sodium pentobarbitone (60mg/kg i.p.) and the hearts removed. The left atrium was isolated and a thread was tied at each end. One end was tied directly to a wire electrode and the other attached via a thread to a Devices isometric transducer. The whole preparation was placed in a 30ml organ bath containing 5.9mM $K^+$ Krebs-Henseleit solution (as previously described)

containing 100$\mu$M ascorbic acid to prevent oxidation of the added noradrenaline, gassed with 95% $O_2$ / 5% $CO_2$. The preparation was placed under a resting tension of 500mg and was stimulated at 50% above the threshold voltage ie. 40-60v, 1ms duration and at a frequency of 0.5Hz, via an electrode adjacent to the muscle and another field electrode placed above the preparation. Atria were stimulated using a Grass SD9 stimulator. Each preparation was allowed 1 hour to equilibrate following which a steady twitch was maintained. Developed tension recordings were made on a Lectromed chart recorder.

**2.1 Effect of P1Pi on the response to noradrenaline in the isolated left atrium, stimulated at 0.5Hz.**

A cumulative concentration-effect curve to noradrenaline was constructed starting at 100nM. The responses were allowed to plateau before the next addition of noradrenaline. The noradrenaline was then completely washed out of the tissue and the preparation allowed 20 minutes to recover. The concentration-effect curve was repeated in the same preparation in order to check for reproducibility. The tissue was washed and in test experiments a concentration of P1Pi was added to the organ bath and left for 20 minutes before repeating the noradrenaline concentration-effect curve. In time-matched control experiments, a third concentration-effect curve was constructed in the absence of P1Pi.

**2.2 Effect of P1Pi on the duration of the twitch response in the isolated, stimulated left atrium in the presence of noradrenaline.**

Left atria were set up as described previously. Following a 30 minute equilibration period, the chart speed was increased from 2.5mm/min to 100mm/sec in order to measure the duration of the twitch response. One peak was recorded before returning

the chart speed to 2.5mm/min. This process was repeated in the presence of 3$\mu$M noradrenaline. The noradrenaline was washed out and the preparation incubated with a concentration of P1Pi before repeating the process in the presence of P1Pi. Time-matched controls were performed in the absence of P1Pi.

**3. Technique for the isolation of rat ventricular myocytes.**

Myocytes were isolated using a modification of the method described by Powell & Twist (1976). Male Wistar rats were anaesthetised with 60mg/kg sodium pentobarbitone and the hearts excised and perfused in a non re-circulating system at 10ml/min using a calcium free salt solution of the following composition (mM):- NaCl 118.5, $NaHCO_3$ 14.5, KCl 2.6, $KH_2PO_4$ 1.18, $MgSO_4$ 1.18, L-glucose 11.1 to pH 7.4, bubbled with 95% $O_2$, 5% $CO_2$. The solution was made up using milli-Q water. The heart was perfused in a non-circulating system until the blood was removed from the coronary vasculature and this perfusate was discarded. The volume of perfusate was adjusted to 40ml and this was allowed to re-circulate the heart. Following 5 minutes of total perfusion time, 20mg Collagenase type II, dissolved in 10ml of the salt solution described above, plus 25$\mu$M $CaCl_2$ were added to the re-circulating solution. Therefore, a final volume of 50ml collagenase solution was re-circulated. After 20 minutes of enzymic digestion the heart was cut down, the atria removed and the ventricles chopped into four pieces using a scalpel and teased apart using forceps. The tissue was then incubated for 10 minutes at 37°C in 20ml of the re-circulating solution bubbled with 95% $O_2$, 5% $CO_2$ and triturated periodically with a pipette. After 10 minutes the mixture was filtered through 250$\mu$M nylon mesh gauze into a centrifuge tube. The cells were spun at 300rpm for 5 minutes and resuspended in the salt solution described above, containing minimal (25$\mu$M) calcium. The cells were washed twice before being resuspended in 10ml solution (25$\mu$M $Ca^{2+}$), HEPES buffered to pH 7.4. The cells were then ready to be loaded with fura-2AM for fluorimetric analysis.

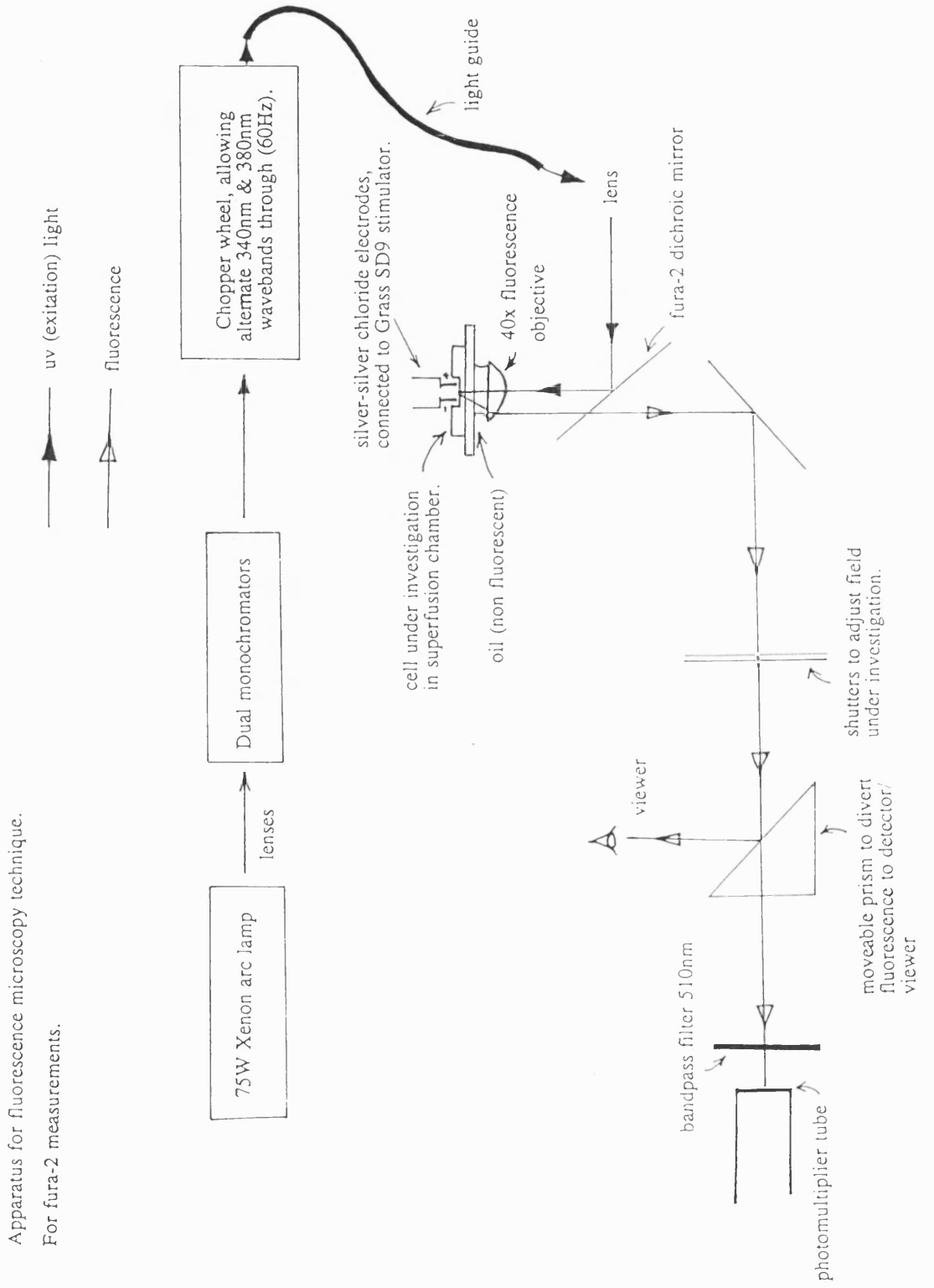
**3.1 Method for the estimation of intracellular calcium in cardiac myocytes using the fluorescent indicator fura-2AM and fluorescence microscopy.**

This technique, first described by Tsien at al. (1985), measures changes in intracellular calcium within a single cell (myocyte), so eliminating problems arising from heterogeneity in a population of cells and the need to correct for signals arising from extracellular dye, which occur in the cuvette system (Li et al., 1987). In addition, single myocytes could be superfused and electrically stimulated whilst measuring changes in intracellular calcium. The fluorescent $Ca^{2+}$ indicator fura-2AM, the acetoxymethyl ester of fura-2 (Grynkiewicz et al., 1985), was used. Upon de-esterification within the cell, fura-2 binds to $Ca^{2+}$ , to increase the 510nm emission from an excitation wavelength of 340nm and decrease the emission from an excitation wavelength at 380nm, which can be used to estimate the free calcium within the cell (see later). This indicator is also very selective for $Ca^{2+}$ over other divalent cations which may be present in the intracellular environment (Grynkiewicz et al., 1985).

The cells were divided into two aliquots of 5 ml. One aliquot was refrigerated at 4°C as reserve. To the remaining 5 ml aliquot was added 2.5$\mu$M fura-2AM, the acetoxymethyl ester of fura-2, and 0.25% BSA to aid loading. The tube was covered with aluminium foil to prevent bleaching of the dye, and cells were loaded for 30 minutes at 37°C. The cells were then spun at 300 rpm for 5 minutes and resuspended in HEPES buffered salt solution containing 100$\mu$M $Ca^{2+}$. The process was repeated, increasing the calcium to 300$\mu$M and finally 1mM. Calcium was added sequentially in order to prevent calcium overload which can occur following rapid addition of a high concentration of calcium. The cells were then left for at least 30 minutes to allow intracellular hydrolysis of the dye before being used for fluorimetry.

**Figure 7**

Technique for the fluorimetric measurement of intracellular calcium in single ventricular myocytes using fura-2.




Apparatus for fluorescence microscopy technique.
For fura-2 measurements.

Myocytes were left at room temperature for at least 30 minutes in order to allow for de-esterification of the acetoxy methyl ester of fura-2 within the cells. They were then placed on a glass coverslip, which formed the base of a perspex superfusion chamber, and which had been pretreated in order to optimise cell adhesion as follows:-

The coverslip was washed, first in alcohol then distilled water, and then polished. A solution of 15$\mu$g/ml laminin (from basement membrane mouse sarcoma) in HEPES buffered salt solution (500ml) was incubated with the coverslip for 1 hour at room temperature. The chamber was then washed thoroughly with fresh HEPES buffered salt solution, and the chamber was left filled with HEPES buffered salt solution until use to prevent the laminin drying. The chamber was placed on the stage of a Nikon Diaphot inverted microscope so that the glass coverslip was in direct contact with the 40x oil-immersion fluorescence objective. The myocytes were left for ten minutes to allow for cell adhesion to the base of the superfusion chamber before being superfused at 2ml/min with 1mM $Ca^{2+}$, HEPES buffered salt solution (see above for composition) at room temperature. The cells were perfused throughout these experiments, to ensure that the concentrations of agonist / antagonist reaching the myocytes was known, as passive diffusion following a bolus addition of agonist / antagonist may result in an non-uniform concentration of drug reaching the myocyte under investigation. The cells were stimulated by means of two silver-silver chloride electrodes, placed at opposite ends of the superfusion chamber. attached to a Grass SD9 stimulator. Myocytes were stimulated at 1Hz, 1ms pulse width, and approximately 50% above the threshold voltage, usually 40-60v. For each experiment a single myocyte was selected using the following criteria:- Clear cross striations, rod shaped with jagged not rounded ends, quiescent prior to electrical stimulation, but showing a consistent contraction in response to each electrical stimulus, well adhered to the coverslip and finally well and evenly loaded with fura-2 (see below).

By diverting the path of light from the objective to a side arm of the microscope, a single myocyte could be isolated by means of vertical and horizontal shutters on the

microscope (see Figure 7). The fura-2 loaded myocyte was superfused and electrically stimulated throughout the experiment. Following approximately 5 minutes superfusion, a UV light source from a 75W arc lamp was passed through the objective to the cell under investigation. This had the effect of exciting the fura-2 within the cell causing it to emit light in the visible spectrum ie. fluoresce. Only cells that were visibly and evenly fluorescent were selected for use. The UV light source consisted of two wavebands of 340 and 380nm from a dual monochromator illuminator source, and these wavebands were transmitted alternately by means of a chopper rotated at 60Hz. The alternating UV wavelengths were transmitted to the myocyte under investigation via a flexible light guide and were reflected off a dichroic mirror before being passed through the oil immersion objective to the glass coverslip holding the myocyte. The resultant fluorescence was transmitted to a photomultiplier tube via a series of lenses and mirrors, and finally to the detector. A 510nm filter ensured that only fluorescence of approximately that waveband was detected. Prior to measurement of fura-2 fluorescence within the myocyte, a background fluorescence was taken. This was done by moving the microscope stage so that the shutters enclosed an area where no myocytes were in view. The 340 and 380 signals were measured in the same way as within the myocyte. The voltage on the detector was then set, in order to obtain a background count of the order $10^3$ photons, usually approximately 510 volts. PTI deltascan software automatically subtracted these background levels whilst collecting and interpreting data from the fura-2 signals produced by the myocyte. With increasing calcium within the cell, the emission at 510nm from the excitation wavelength of 340nm (1A) will increase, and from the excitation wavelength of 380nm (2A) the emission at 510nm will decrease. Hence the 340/380 signal ratio is a function of the concentration of free calcium within the cell. The signal ratio was measured, as opposed to just the emission from an excitation wavelength at 340nm as this allows an estimation of the calcium concentration within the cell independent of cell loading. Additionally, it eliminates changes in the signal occurring as a result of changes in intensity of the excitation light, leakage or bleaching of the intracellular dye, all of which could be interpreted

as a decrease in intracellular calcium, as this will cause a decrease in both the 1A and 2A signal. It also eliminates changes occurring due to cell 'thickening' occurring during a twitch, as this will cause an increase in both the 1A and 2A signal (Capogrossi & Lakatta, 1989). This ratio was sampled 60 times every minute, the rate being controlled by the rate of rotation by the chopper. 340/380 signal ratios were used to give an estimate of relative changes in calcium concentration within the myocyte.

The concentration of calcium within the cell was not calibrated due to the well established difficulties with this technique ie. ion binding and spectral behaviour in the intracellular environment (Capogrossi & Lakatta, 1989; Frampton et al., 1991) and incomplete hydrolysis of ester groups within the cell, leaving calcium insensitive species (Scanlon et al., 1987), and spectral interference with intrinsic fluorescent compounds such as NADPH (Koretsky et al.,1987). Even using intracellular calibration creates difficulties in the case of myocytes, due to the hypercontracture which develops on exposure to saturating levels of calcium (Li et al., 1987; Borzak et al., 1991) and compartmentalisation of the dye into intracellular organelles eg. mitochondria (Steinberg et al., 1987), which may produce a false indication of cytosolic free calcium. Additionally, we were interested in seeing changes in the peak heights of calcium oscillations in response to agonists rather than actual calcium concentrations.

Typically, signals of between $10^5$ and $10^6$ counts per second were obtained for the 1A and 2A signals, representing the emission at 510nm from excitation wavelengths of 340nm and 380nm respectively ie. approximately 10 times above the background signal. An estimate of intracellular free calcium was obtained by obtaining the 340/380 signal ratio, which is directly proportional to the calcium concentration within the myocyte. The calcium transients in response to electrical stimulation at 1Hz varied greatly in different individual myocytes, probably due to differences in cell size and original location within the ventricles. Therefore a large sample number

was obtained for each experiment in order to estimate the average increase in the 340/380 signal ratio in response to electrical stimulation at 1Hz. In order to standardise the experiments, data representing the mean of all cells under investigation was obtained from the mean of five peaks from the 50-55 seconds interval on the time scale for each myocyte. Responses in the presence of agonist were measured at 150-155 seconds on the time scale.

### 3.1.1 Effect of noradrenaline, verapamil or caffeine on the 340/380 fura-2 signal ratio increase in response to electrical stimulation at 1Hz in a single myocyte.

Cells were isolated, loaded with fura-2AM and prepared for fluorimetry as described above. Cells were allowed to settle for 10 minutes then perfused and stimulated at 1Hz, as described above, for another 5 minutes before any fluorimetric measurements were taken. Following this, the illuminating UV source was deflected, to illuminate the myocyte under investigation (this was left until just before measurement to prevent bleaching of the dye) and measurement commenced. Basal 340/380 signal ratio oscillations were taken for 1 minute prior to the addition of noradrenaline, verapamil or caffeine. Following this, a concentration of noradrenaline, verapamil or caffeine was included in the perfusate and measurement was continued for 3 minutes. After measurement, the superfusion chamber was washed thoroughly and a new batch of cells were used for each subsequent experiment.

### 3.1.2 Experiment to investigate the direct effect of P1Pi on the 340/380 signal ratio increase in response to electrical stimulation at 1Hz in a single myocyte.

Separate experiments were carried out to discover whether P1Pi had a direct effect on myocytes. The chamber was superfused with HEPES buffered salt solution in the absence of P1Pi for 5 minutes, as described previously and a 340/380 signal ratio

measurement taken, before adding a concentration of P1Pi to the perfusate. The preparation was superfused for 5 minutes in the presence of P1Pi before a measurement of the 340/380 signal ratio in the presence of P1Pi was taken.

### 3.1.3 Effect of noradrenaline in the presence of P1Pi or atenolol on the 340/380 signal ratio increase in response to electrical stimulation at 1Hz in a single myocyte.

These experiments were carried out using a similar protocol to that described in section 3.1.1, except cells were superfused for 5 minutes prior to the commencement of fura-2 measurement, and throughout the experiment, in the presence of a concentration of P1Pi or atenolol. The time course of the experiment was therefore kept the same. The same protocol was used to investigate the effect of P1Pi on caffeine responses.

## 4. Drugs and materials.

P1Pi and all synthetic derivatives of palmitoyl carnitine were synthesized by M. Rad-Niknam and G.H. Dewar in the School of Pharmacy and Pharmacology laboratories, University of Bath.

Palmitoyl carnitine, isobutyl methyl xanthine (IBMX), sodium nitroprusside, papaverine, tetraethylammonium chloride (TEA), atenolol, polymyxin B, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), isoprenaline, caffeine, methoxamine, noradrenaline, bovine serum albumin (BSA) and laminin (basal membrane mouse sarcoma). All purchased from Sigma.

Also used : Verapamil (Abbot), salbutamol (Allen and Hanbury), lemakalim (SmithKline Beecham), nicardipine (Syntex), BAY K 8644 (Bayer), collagenase type II (Worthington), fura-2AM (Molecular probes).

All compounds were first dissolved in 0.9% saline or PSS except BAY K 8644, IBMX, fura-2AM (DMSO), $PGF_{2\alpha}$ (ethanol) and lemakalim (66% ethanol, 33%$H_2O$) subsequent dilutions being made in saline / PSS. All solutions of noradrenaline had 100$\mu$M ascorbic acid added to prevent oxidation.

## 5. Statistical analysis.

All data is expressed as mean$\pm$sem.

Differences between means were assessed using either a paired or unpaired Student's t-test, or a one or two way analysis of variance (ANOVA) followed by a Studentised range test or Dunnett's test, as appropriate. A p value of less than 0.05 was considered significant (indicated by * on figures).

## Results

### 1.1 Structure-activity relationship studies on novel synthetic acyl carnitine derivatives.

Structure-activity relationship studies were carried out in order to elucidate which groups of the molecule of the coronary dilator derivatives of palmitoyl carnitine are important in eliciting the coronary dilator activity, ie. whether the positive charge on the quaternary nitrogen or the ester grouping attached at the C terminus of the carnitine moiety are important in eliciting a coronary dilator response.

For all the structure-activity relationship studies combined, the perfusion pressure in the presence of 5.9mM $K^+$ Krebs-Henseleit was 70±2.9mmHg, n=69. On switching to modified Krebs-Henseleit containing 3.2mM $K^+$, the perfusion pressure increased to a mean of 97±2.6mmHg ie. an average increase in perfusion pressure of 27mmHg. This allowed a greater scope for coronary dilator responses to be seen.

#### 1.1.1 Effect of removing the terminal ester group on coronary dilator activity.

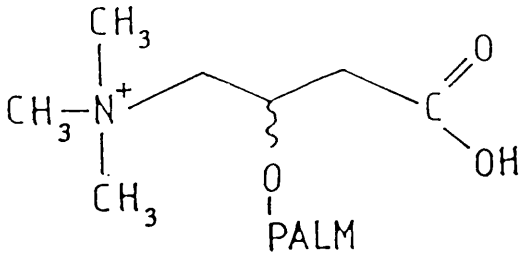
The isopropyl ester of palmitoyl carnitine (P1Pi) differs from the parent compound palmitoyl carnitine by the presence of an isopropyl ester group in place of a carboxylic acid group on the terminal carbon (Table 1). Whereas palmitoyl carnitine is a potent constrictor in the coronary vasculature (Criddle et al., 1990) it can be seen from Figure 8 that P1Pi is a potent vasodilator. Figure 8 is a typical trace showing the effect of P1Pi on perfusion pressure in the isolated perfused heart preparation in the presence of 3.2mM $K^+$. Bolus doses of P1Pi produced a long lasting coronary dilator effect, the maximum dilation being produced at 3nmoles. There was no concomitant effect on developed tension or heart rate.

Figure 9 is a representative trace showing the effect of compound A, which has a similar structure to P1Pi but with an ethyl group in the place of the isopropyl ester grouping (Table 1), on perfusion pressure in the presence of 3.2mM $K^+$. It can be seen that this compound also produces coronary dilation in the Langendorff isolated perfused heart. In addition, this compound produced a transient variable coronary constriction preceding the coronary dilator effect. Figure 10 compares dose response curves to P1Pi and Compound A. It can be seen from this graph that at lower doses (up to 3nmoles) Compound A produced significantly ($p < 0.05$) smaller coronary dilator responses than P1Pi. However, on comparing $ED_{50}$'s and maximum coronary dilator responses (Table 3), there was no significant difference between P1Pi and Compound A. However, it is clear from Figures 8 and 9 that bolus doses of P1Pi produced a longer lasting coronary dilator effect than Compound A.

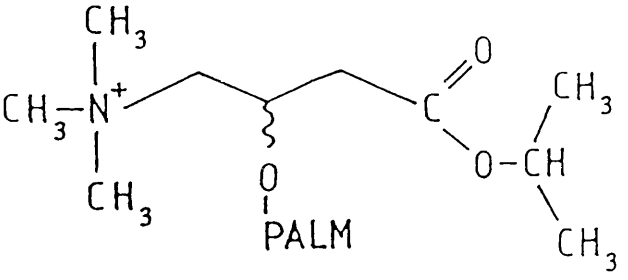
Table 1

Structures of the palmitoyl carnitine derivatives used in this study.

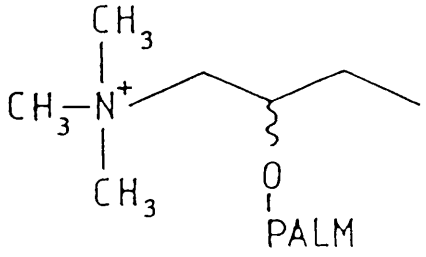
Palmitoyl carnitine



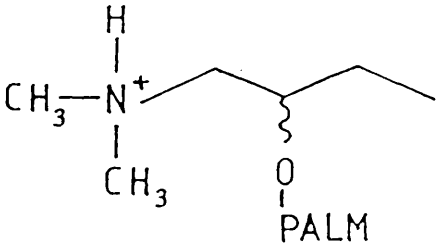

Palmitoyl carnitine Isopropyl ester (P1Pi)



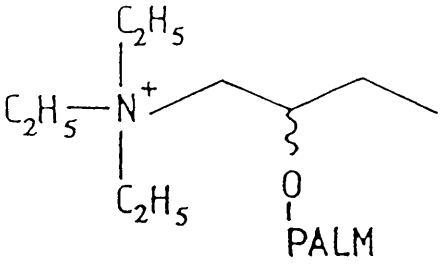

Compound A



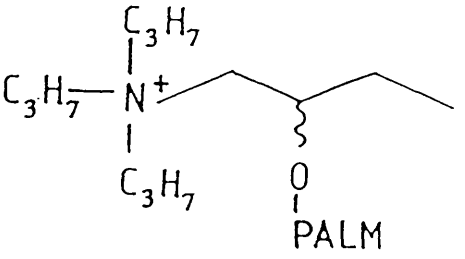

Compound B



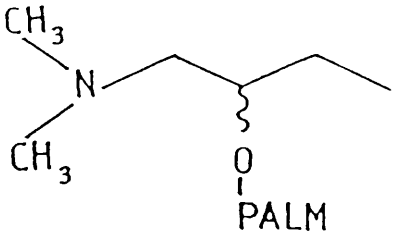

Compound C




Compound D




Compound E




Compound F

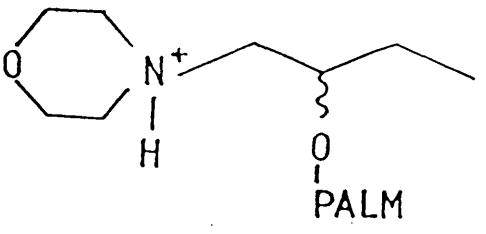
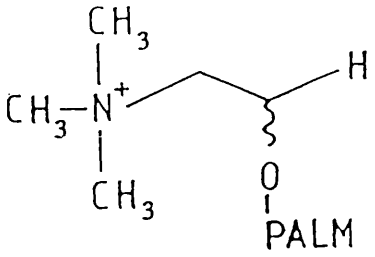
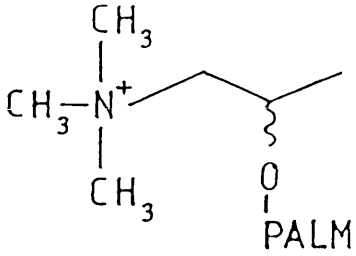


Table 1 cont.

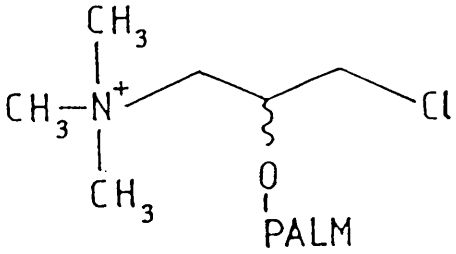
Compound G



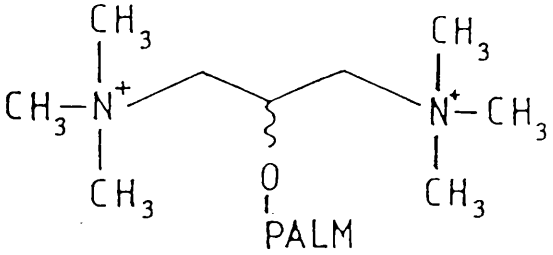

Compound H



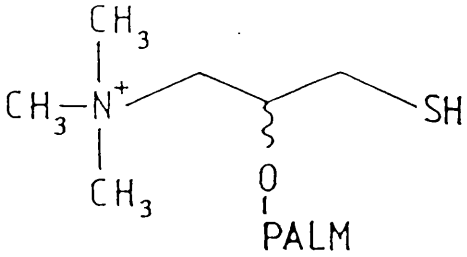

Compound I



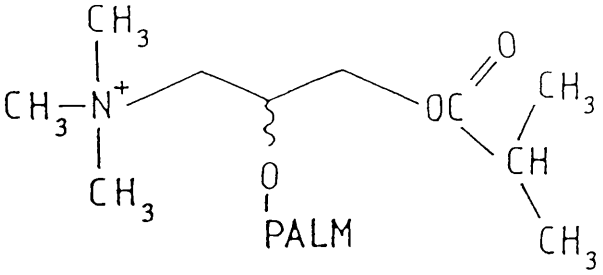

Compound J



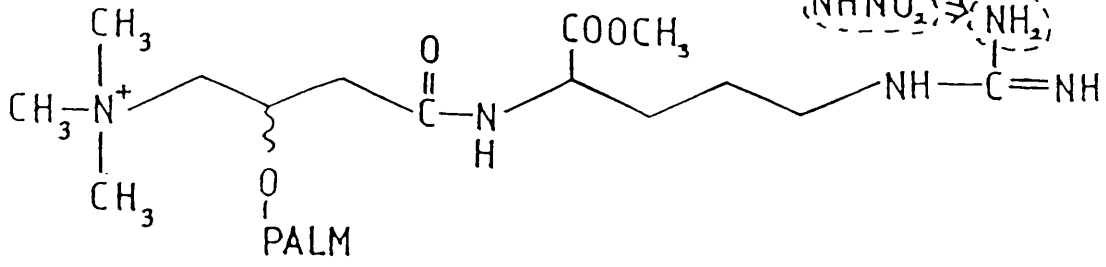

Compound K




Compound L




Compound M




Compound N

Figure 8

Trace showing the effect of bolus doses of P1Pi on coronary perfusion pressure in the isolated perfused rat heart.

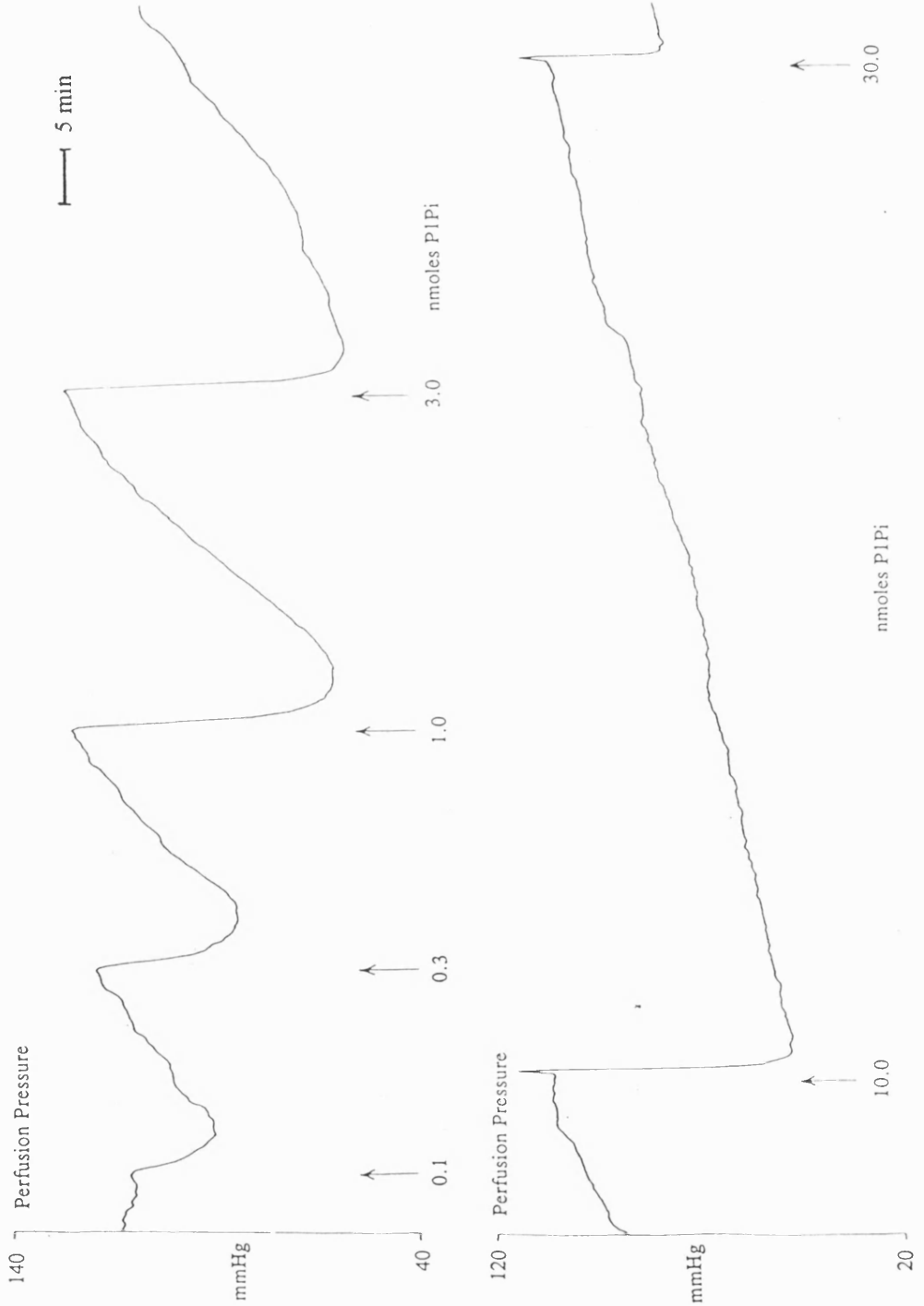


Figure 9

Trace showing the effect of bolus doses of compound A on coronary perfusion pressure in the isolated perfused rat heart.

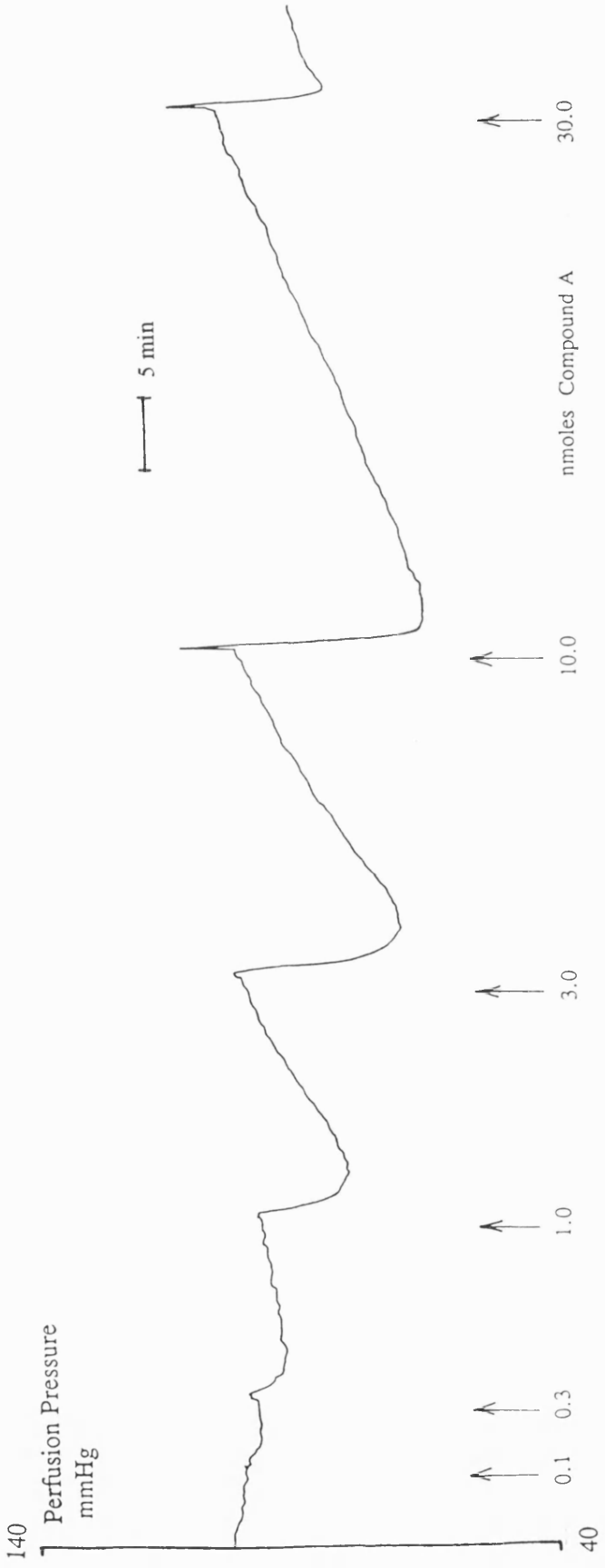
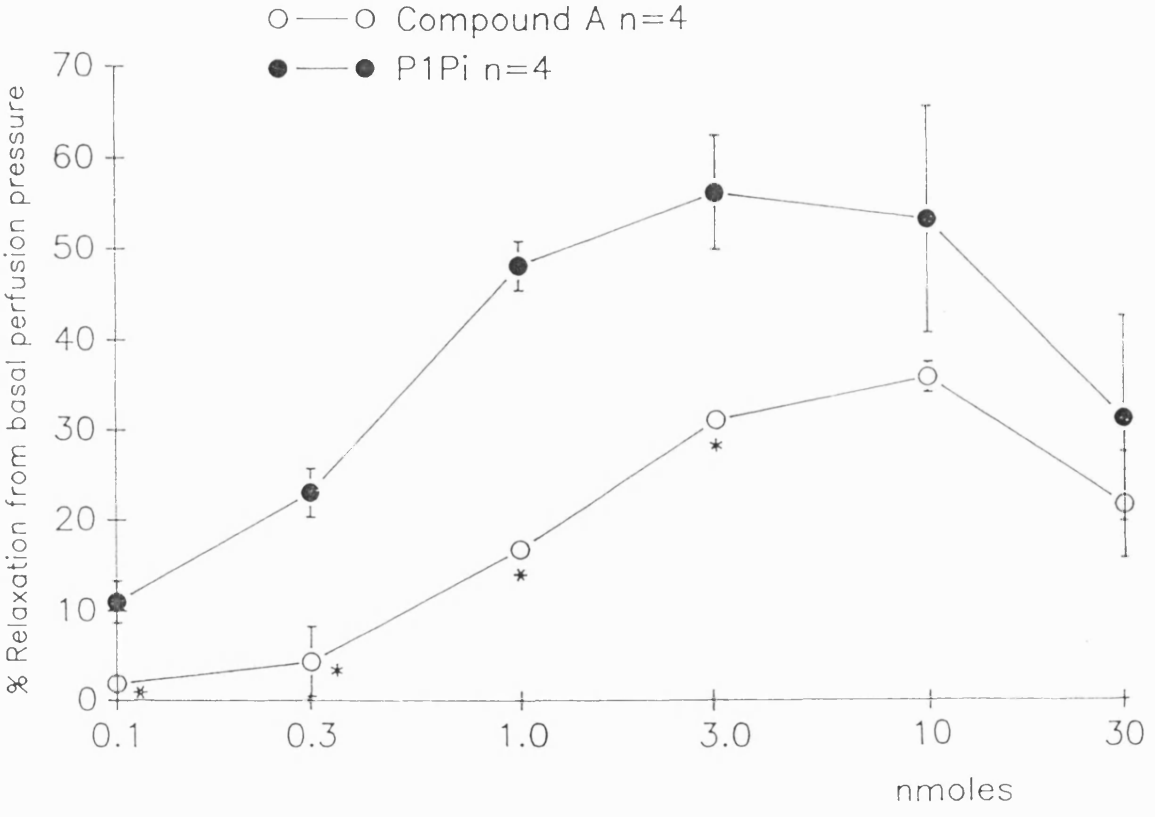


Figure 10

The effect of P1Pi and Compound A (see Table 1) on perfusion pressure in the isolated perfused rat heart. Data expressed as % relaxation from basal (elevated) perfusion pressure.

Basal perfusion pressure: P1Pi 114±3.1mmHg n=4; Compound A 101±4.1mmHg n=4. *$p < 0.05$, Student's unpaired t-test.



### 1.1.2 Effect of N terminus substitution on coronary dilator activity.

Figure 11 and Table 2 compare the potencies of compounds all containing the same C terminus substitution (ethyl group) but differing in their N terminus substitution. The $ED_{50}$'s and maximum dilator responses were all compared with Compound A, with three methyl groups attached to the nitrogen, used as a reference compound. Compound C, containing three ethyl groups attached to the nitrogen, was significantly more potent than Compound A (indicated by a significantly lower $ED_{50}$ ;$p < 0.05$). Compound B, containing two methyl groups and a hydrogen, was both significantly less potent and less effective than Compound A (indicated by a significantly higher $ED_{50}$ and significantly lower maximum dilator response ($p < 0.05$). Compound D, containing three propyl groups at the N terminus, was not significantly different to Compound A in terms of potency or maximum dilation achieved.

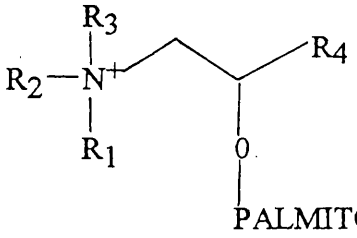
It can also be seen from Figure 11 and Table 2 that Compound F, containing a morpholinyl ring in place of alkyl groups at the N terminus (Table 1) is the least potent compound of all. An $ED_{50}$ was not obtainable for this compound due to much variability in the dilation achieved in different preparations. However, the maximum dilator response was significantly lower than that of Compound A. Compound E contains an uncharged terminus nitrogen, due to only two methyls being attached (Table 1). It can be seen from Figure 11 that this compound is completely inactive as a coronary dilator, and produced a variable constrictor response only. All compounds tested showed a transient coronary constrictor response preceding dilation, similar to that seen for compound A in Figure 9.

Table 2.

Effect of N terminus ($R_1$, $R_2$ & $R_3$) substitution on vasodilator activity, while keeping C terminus constant, ($R_4$=Et).

| Comp. | n= | $R_4$ | $R_1$ | $R_2$ | $R_3$ | Basal pp (mmHg) | $ED_{50}$ nmol. | Max % Dil. (Dose) |
|---|---|---|---|---|---|---|---|---|
| A | 4 | Et | Me | Me | Me | 101±4.1 | 1.06±0.07 | 36±1.8 (10nmol) |
| B | 4 | Et | H | Me | Me | 78±8.8 | 12.8±2.5* | 15±1.4 (30nmol)* |
| C | 4 | Et | Et | Et | Et | 83±10.6 | 0.26±0.09* | 35±8.0 (1nmole) |
| D | 4 | Et | Pr | Pr | Pr | 117±6.6 | 0.60±0.13 | 36±3.7 (10nmol) |
| E | 6 | Et | Me | Me | -- | 110±10.7 | --- | INACTIVE |
| F | 4 | Et | H | | O | 109±8.3 | --- | 10±3.3 (100nmol)* |

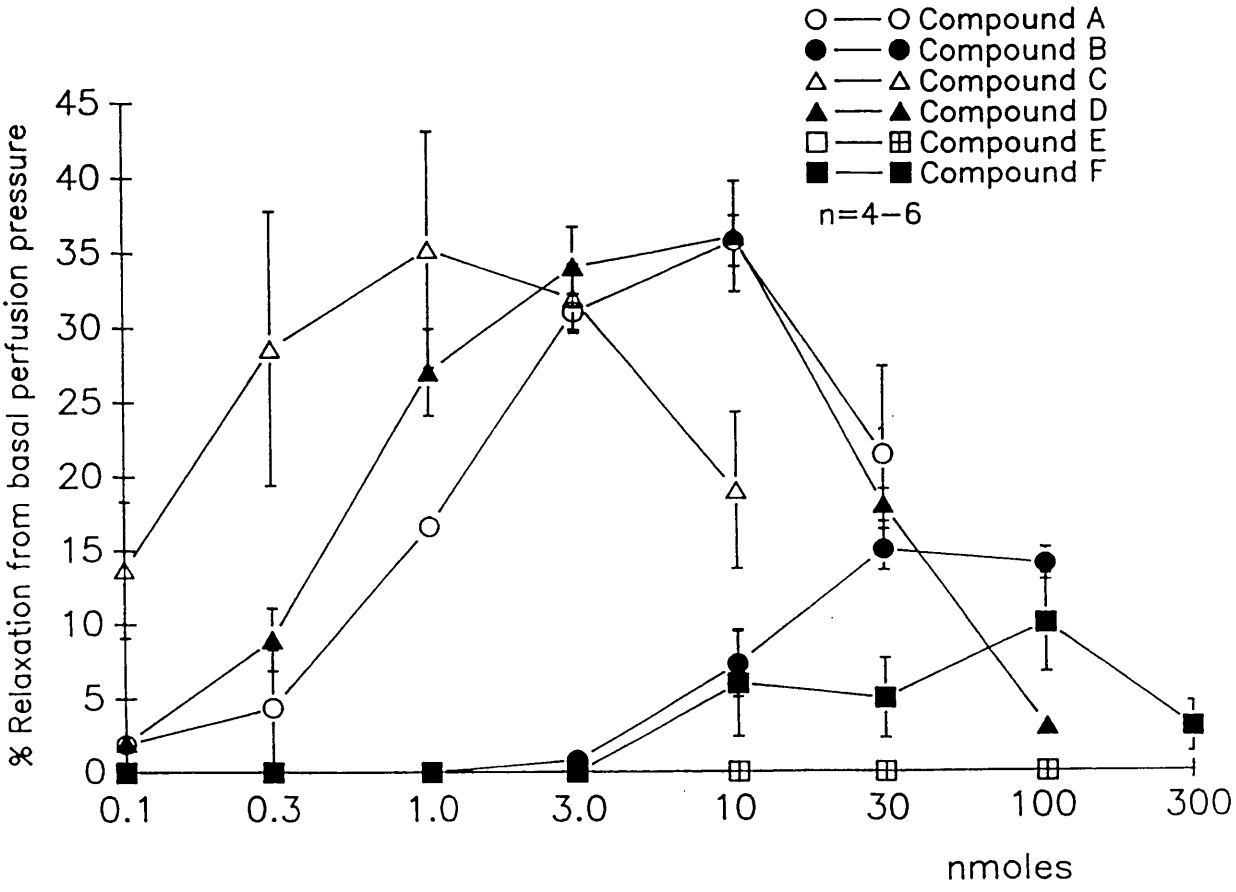
For $ED_{50}$ and Max % Dil. *$p<0.05$,. compared with Compound A (1way ANOVA, followed by Dunnett's test, for many comparisons to one Control group, ie. Compound A).
Max % Dil.= The % relaxation from basal perfusion pressure at the optimal dose.
Me=methyl
Et=ethyl
Pr=isopropyl
pp=Perfusion pressure




(N.B. Compound E is uncharged)

Figure 11

Graph comparing the coronary dilator potencies of Compounds A, B, C, D, E, F (see Tables 1 & 2), differing in their N terminus substitution, in the isolated perfused rat heart. Data expressed as % relaxation from basal (elevated) perfusion pressure. (See Figure 12 for basal perfusion pressures).



### 1.1.3 Effect of C terminus substitution on coronary dilator activity.

Table 3 and Figures 12a & 12b illustrate the effect of C terminus substitution on coronary dilator activity, whilst keeping the N terminus constant ie. three methyl groups. All compounds were compared with Compound A, containing an ethyl group at the C terminus, in terms of $ED_{50}$ values and maximum dilation achieved. All compounds containing the same N terminus and differing in their C terminus were equieffective in terms of maximum dilation achieved. In addition, C terminus substitution appeared to have a less profound effect on coronary dilator potency than N terminus substitution. However, Compounds G (hydrogen in place of a C terminus), J (containing a second quaternary nitrogen grouping), M and N (containing arginine and nitroarginine moieties respectively) were all significantly ($p < 0.05$) less potent, indicated by higher $ED_{50}$ values (Table 3).

Compound L, an isomer of P1Pi, Compound H, containing a methyl group at the C terminus, Compound I, containing a chloromethyl and Compound K, containing a thiol group were all found to be not significantly different to Compound A in terms of coronary dilator potency.

As for the compounds in Table 2, all the compounds in Table 3 showed a transient coronary constrictor effect preceding the coronary dilation. This was less marked in the case of P1Pi and its isomer Compound L. Compound G containing a single hydrogen atom at the C terminus produced the greatest coronary constriction, producing at the optimum dose (30nmoles) a constriction of 24.7±5.3mmHg (Figure 14).

Table 3

Effect of C terminus ($R_4$) substitution on vasodilator activity, whilst keeping $N^+$ terminus constant ($R_1$, $R_2$ & $R_3$= 3x Me).

| Comp. | n= | $R_1$, $R_2$ &$R_3$ | $R_4$ | Basal pp (mmHg) | $ED_{50}$ nmol | Max % Dil (Dose) |
|---|---|---|---|---|---|---|
| A | 4 | 3x Me | Et | 101±4.1 | 1.1±0.07 | 36±1.8 (10nmol) |
| P1Pi | 4 | 3x Me | $CH_2COOC_3H_7$ | 114±3.1 | 0.44±0.09 | 56±6.3 (3nmol) |
| G | 6 | 3x Me | H | 95±5.6 | 5.4±1.0* | 42±8.8 (30nmol) |
| H | 6 | 3x Me | Me | 77±9.7 | 1.6±0.3 | 24±4.0 (10nmol) |
| I | 4 | 3x Me | $CH_2Cl$ | 88±6.5 | 2.9±1.0 | 37±4.5 (10nmol) |
| J | 3 | 3x Me | $CH_2N^+Me$ | 113±14.8 | 8.5±3.1* | 45±12.5 (100nmol) |
| K | 4 | 3x Me | $CH_2SH$ | 83±11.1 | 3.3±1.3 | 27±8.6 (30nmol) |
| L | 4 | 3x Me | $CH_2OCOC_3H_7$ | 102±10.9 | 0.58±0.03 | 42±5.4 (3nmol) |
| M | 5 | 3x Me | $CH_2CO$-ARG | 110±8.6 | 7.1±1.4* | 59±4.6 (30nmol) |
| N | 5 | 3x Me | $CH_2CO$-NOARG | 96±11.1 | 3.9±1.0* | 41±4.9 (30nmol) |

For $ED_{50}$ and Max % Dil. *$p<0.05$,. compared with Compound A (1way ANOVA, followed by Dunnett's test, for many comparisons to one Control group, ie. Compound A).
Max % Dil.= The % relaxation from basal perfusion pressure at the optimal dose.
Me=methyl
ARG=L-arginine
NOARG=L-nitroarginine
pp=Perfusion pressure

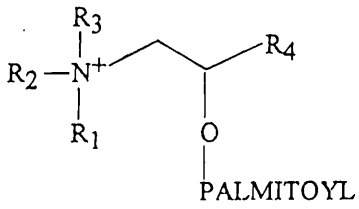


Figure 12a

Graph comparing the coronary dilator potencies of compounds differing in their C terminus substitution in the isolated perfused rat heart. Data expressed as % relaxation from basal (elevated) perfusion pressure.

Figure 12a: Compounds A,G,H,I. (See Tables 1 & 3).

Mean basal perfusion pressure for all compounds tested 96±2.6mmHg n=69.

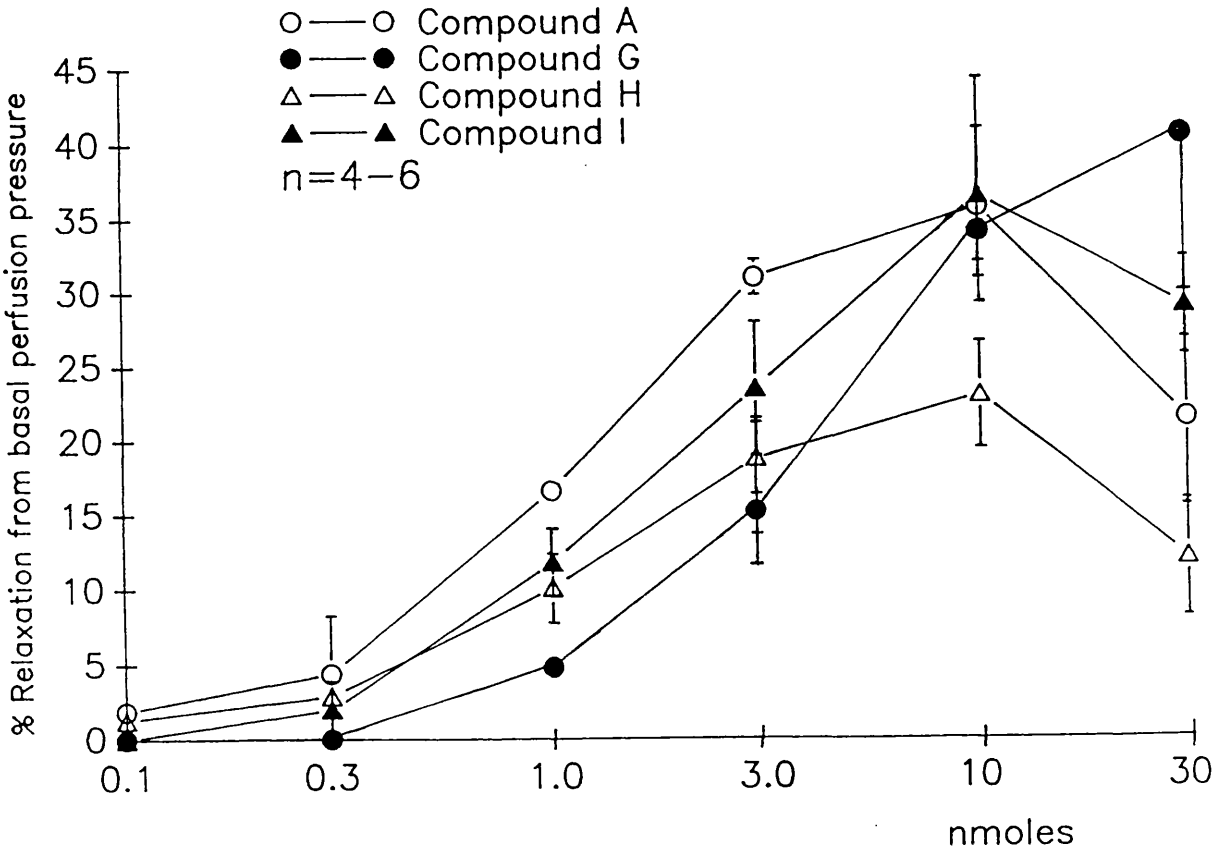
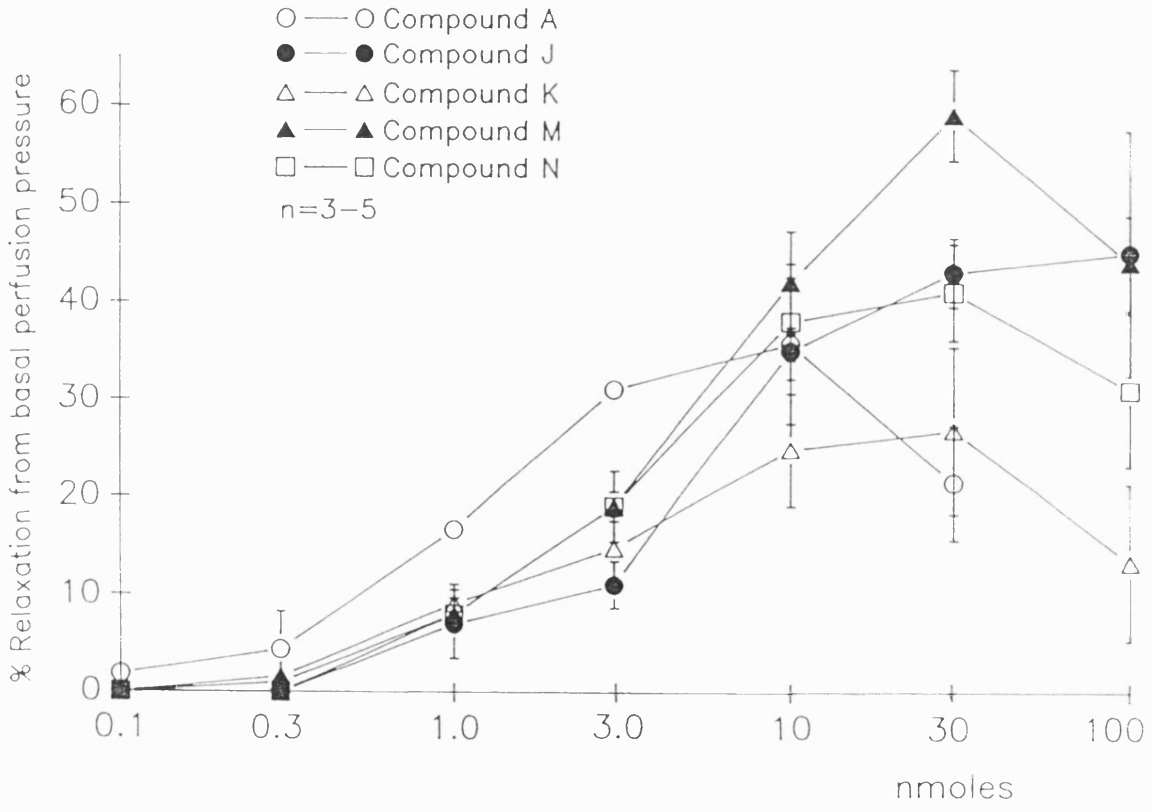


Figure 12b

Graph comparing the coronary dilator potencies of compounds differing in their C terminus substitution in the isolated perfused rat heart. Data expressed as % relaxation from basal (elevated) perfusion pressure.

Figure 12b: Compounds A,J,K,M,N. (See Tables 1 & 3).

Mean basal perfusion pressure for all compounds tested 96±2.6mmHg n=69.



## 1.2 Effect of verapamil on constrictor and dilator responses to Compound G.

The effect of verapamil on responses to compound G was investigated in order to investigate whether the coronary constrictor or the coronary dilator activity of compound G is mediated via an effect on L-type calcium channels.

Compound G was used for these experiments as it produced the greatest and most consistent degree of coronary constriction, as well as a coronary dilator response. It can be seen from Figure 13 that the constrictor component of the response to compound G is almost completely lost in the presence of 100nM verapamil. This is shown also in the graph in Figure 14. The coronary dilator component was also significantly reduced ($p < 0.05$), at the optimal dose (30nmoles) it was reduced from $42 \pm 8.8\%$ to $4.5 \pm 2.6\%$. However, verapamil itself significantly reduced the basal perfusion pressure; $95 \pm 5.6$mmHg in the absence of verapamil and $53 \pm 2.4$mmHg in the presence of verapamil (100nM; $p < 0.05$).

Figure 13

Trace showing the effect of 100nM verapamil on the coronary dilator and constrictor responses to bolus doses of Compound G in the isolated, perfused rat heart.

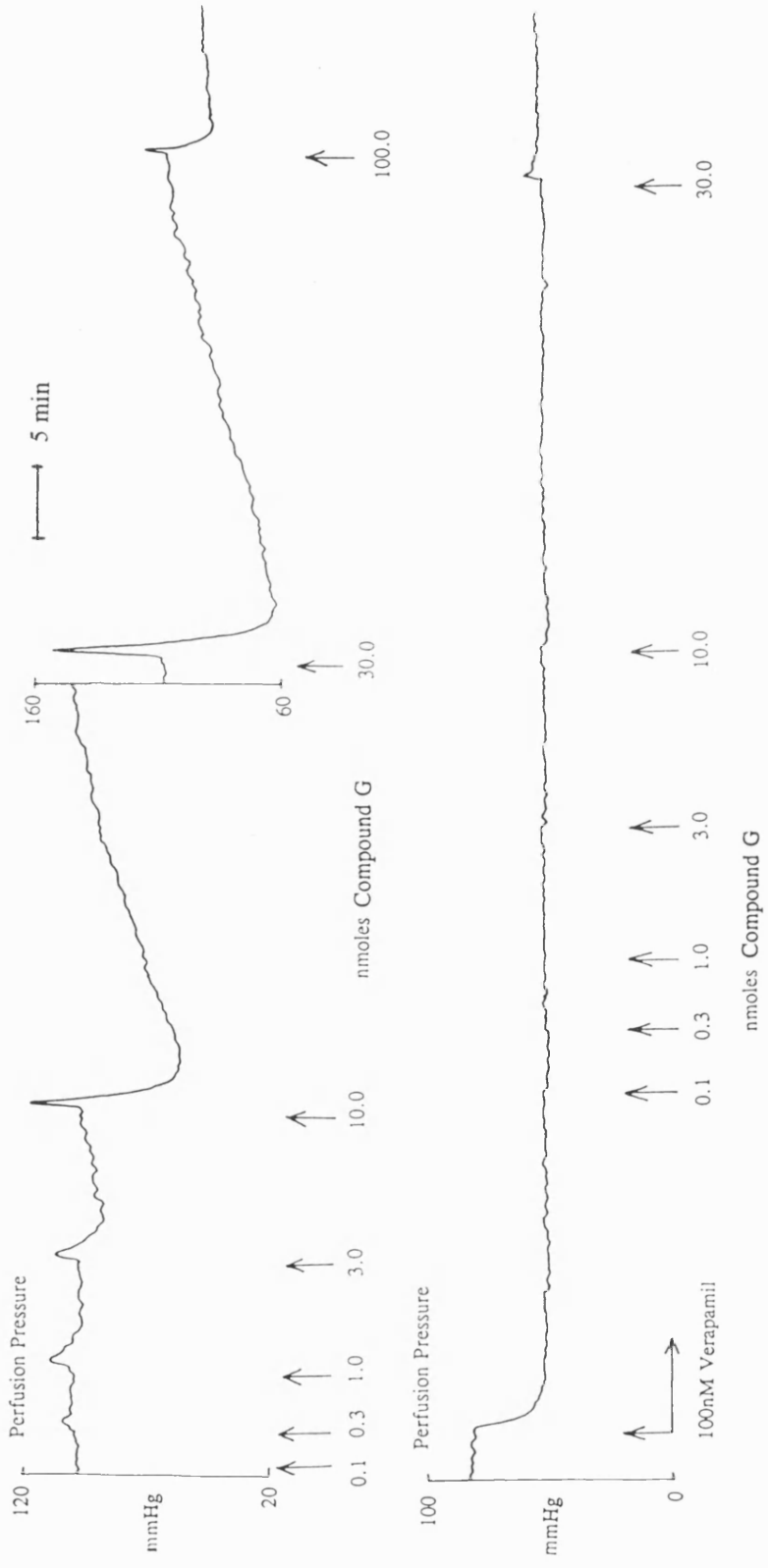
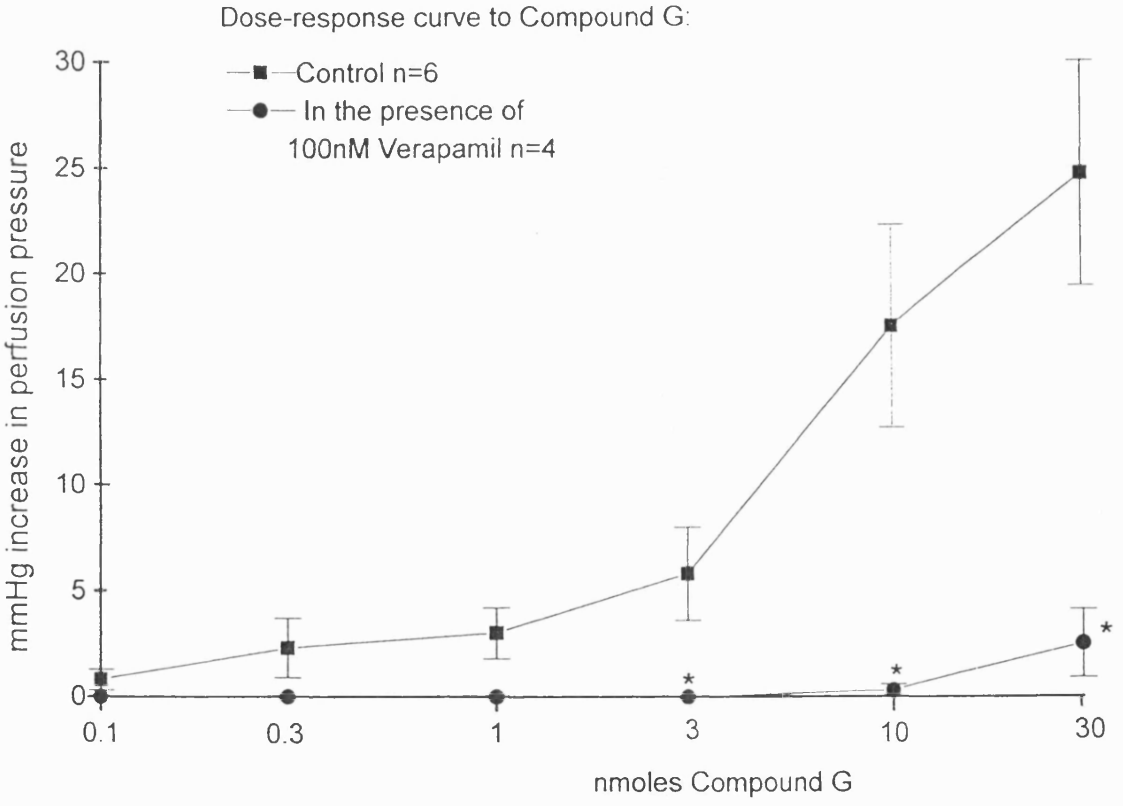


Figure 14

The effect of 100nM verapamil on coronary constrictor responses to bolus doses of Compound G in the isolated, perfused rat heart. Data expressed as mmHg increase in perfusion pressure.

Basal perfusion pressure: Control 95±5.6mmHg n=6; In presence of 100nM verapamil 53±2.4mmHg n=4. *$p < 0.05$, Student's unpaired t-test.



### 1.3.1 Effect of 110mM $K^+$ on coronary dilator responses to P1Pi, verapamil, papaverine, salbutamol, nicardipine and lemakalim.

These experiments were performed to investigate a possible action of P1Pi on potassium channels. A concentration of 110mM $K^+$ outside the coronary smooth muscle cells produces a depolarisation of the smooth muscle and hence coronary constriction.

According to the Nernst equation (At body temperature (37°C)), and assuming the membrane to be permeable only to potassium:-

$$E_m = 61 \quad .\log_{10} \frac{[\text{Conc. of } K^+ \text{ outside the cell}]}{[\text{Conc. of } K^+ \text{ inside the cell}]}$$

$E_m$ being the potential difference across the sarcolemma.

Therefore, prior to the addition of high potassium ie. in the presence of 3.2mM $K^+$, according to the Nernst equation:-

$$E_m = 61 \quad .\log_{10} \frac{[3.2]}{[140]} = -100mV$$

Whereas on perfusion of 110mM $K^+$ solution:-

$$E_m = 61 \quad .\log_{10} \frac{[110]}{[140]} = -6mV$$

However, in the presence of 3.2mM $K^+$ the potential across the sarcolemma is likely to be much less negative than the membrane potential produced by the Nernst equation, as at this concentration physiologically, other factors such as ion pumps can affect the membrane potential. A concentration as low as 3.2mM. $K^+$ in coronary

smooth muscle is likely to slightly depolarise the smooth muscle; because in these experiments, switching to 3.2mM $K^+$ from an original 5.9mM $K^+$ perfusate produced an increase in coronary perfusion pressure (see Section 1.1, Results). This is possibly due to a low concentration of $K^+$ ions on the outer sarcolemmal surface producing an inhibition of $Na^+$-$K^+$-ATPase, thus producing an increase in intracellular sodium. This could cause a secondary increase in intracellular calcium through an increase in calcium influx through the $Na^+$-$Ca^{2+}$ exchanger and subsequent calcium-induced calcium release from the sarcoplasmic reticulum. Thus, the Nernst equation cannot give an accurate estimate of the membrane potential at this low concentration of potassium. However, from the Nernst equation, it is clear that in the presence of 110mM $K^+$, there is a very high degree of depolarisation of the membrane potential, to -6mV. This has been shown to impair the action of vasodilators which act via activation of potassium channels, as a high concentration of potassium outside the cells will reduce the driving force of potassium ions out of the cell following potassium channel activation (Hamilton et al., 1986; Weir and Weston 1988). The effect of 110mM $K^+$ on the actions of other coronary dilators, known to act either independently of potassium channels (verapamil, papaverine, nicardipine) or via the activation of potassium channels (lemakalim and possibly salbutamol), were compared with the effect of 110mM $K^+$ on the coronary dilator effect of P1Pi. The dose used for each coronary dilator was chosen to produce an approximately equivalent degree of coronary dilation.

Figure 15 is a representative trace showing the effect of 110mM $K^+$ on the coronary dilator responses to verapamil and P1Pi. Following perfusion of 110mM $K^+$, a contracture developed in the myocardium together with an increase in perfusion pressure (Before:- $108 \pm 3.2$mmHg, in presence of 110mM $K^+$:- $154 \pm 4.9$mmHg, $n=50$), however there was no significant difference in the response to a submaximal dose of verapamil (1nmole), before or during perfusion with 110mM $K^+$. Prior to 110mM $K^+$ perfusion:- $43 \pm 1.5$mmHg; in presence of 110mM $K^+$:- $37 \pm 2.3$mmHg dilation, $n=50$. The 1nmole verapamil dose was used to gauge responses to other

vasodilators shown in Figure 16. Each coronary dilator dose was repeated to ensure reproducibility prior to and following the perfusion of 110mM $K^+$, indicated by the two bars on the graph before and after treatment. These were administered alternately with 1nmole of the calcium antagonist verapamil, used as an internal control. It can be seen from Figure 16 that P1Pi (0.5nmoles) produced a coronary dilator response that was reproducible in the same preparation and approximately equal to a 1nmole verapamil response in terms of degree of dilation. The P1Pi response was virtually abolished following perfusion with 110mM $K^+$ ($p<0.05$; Figure 16). Coronary dilator responses produced by 10nmoles of the $K_{ATP}$ channel activator lemakalim were completely abolished in the presence of 110mm $K^+$. Papaverine (30nmoles) and the dihydropyridine nicardipine (0.5nmoles) responses were not significantly altered in the presence of 110mM $K^+$ (NB. The 0.5nmole nicardipine dose was not repeated prior to administration of 110mM $K^+$, due to the long duration of the coronary dilator response). Salbutamol responses were smaller ie. not equivalent to 1nmole verapamil, but were significantly reduced in the presence of 110mM $K^+$.

In time-matched control preparations (Figure 17), verapamil responses were not significantly different before or after 15 minutes control perfusion ($35\pm3.5$mmHg initially, $32\pm5.0$mmHg after time-matched perfusion period, $n=18$). P1Pi and lemakalim responses were also not significantly different following 15 minutes time-matched perfusion. However, there was a significant ($p<0.05$) tachyphylaxis to the salbutamol response.

Figure 15

Trace showing the effect 110mM potassium on the coronary dilator, developed tension and heart rate responses to P1Pi and verapamil in the isolated perfused rat heart.

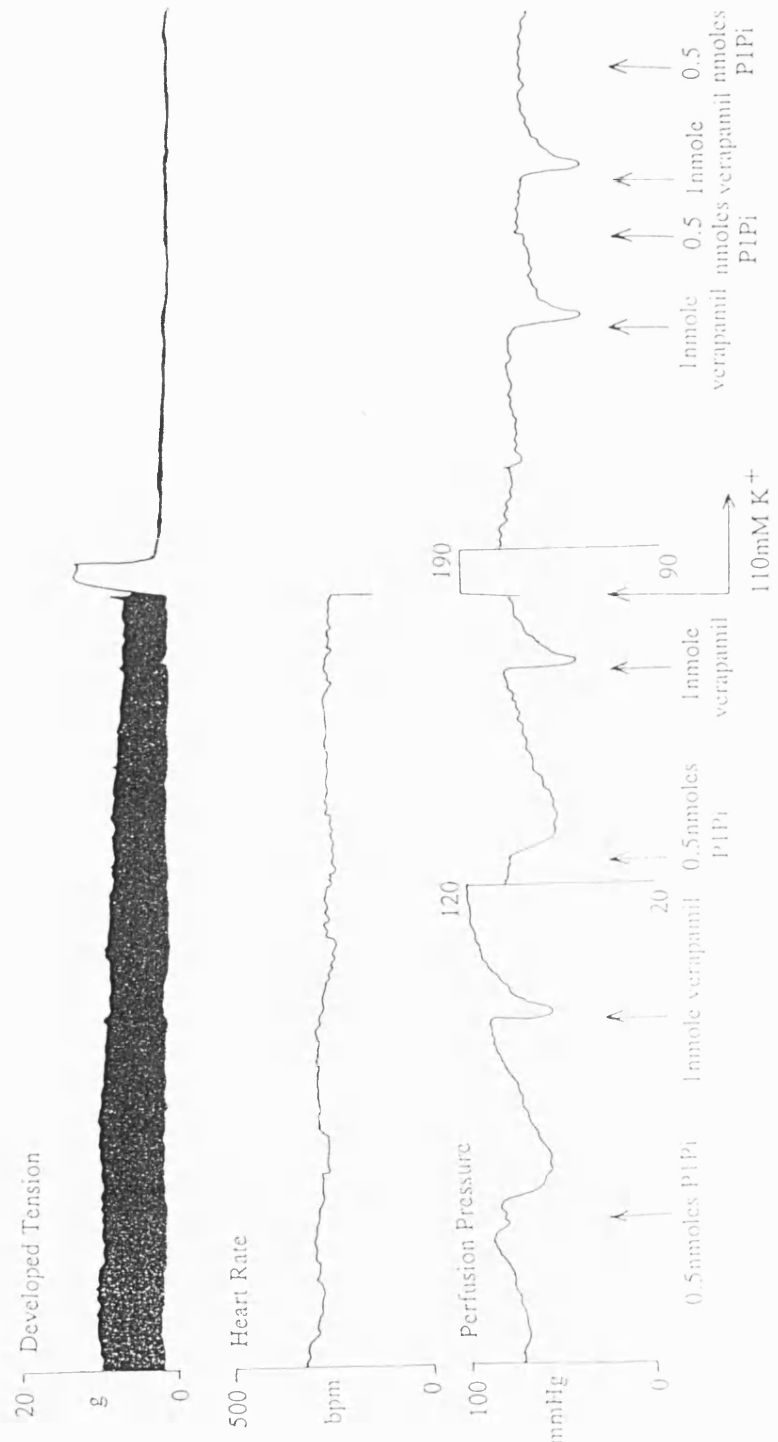


Figure 16

Effect of 110mM potassium on the coronary dilator responses to bolus doses of P1Pi, lemakalim, papaverine, salbutamol and nicardipine in the isolated perfused rat heart. Each dose was repeated to ensure reproducibility, except in the case of nicardipine. Data expressed as % 1nmole verapamil response.

Basal perfusion pressure: 3.2mM $K^+$ 107±3.2mmHg; 110mM $K^+$ 154±4.9mmHg n=18. *$p < 0.05$ Student's paired t-test.

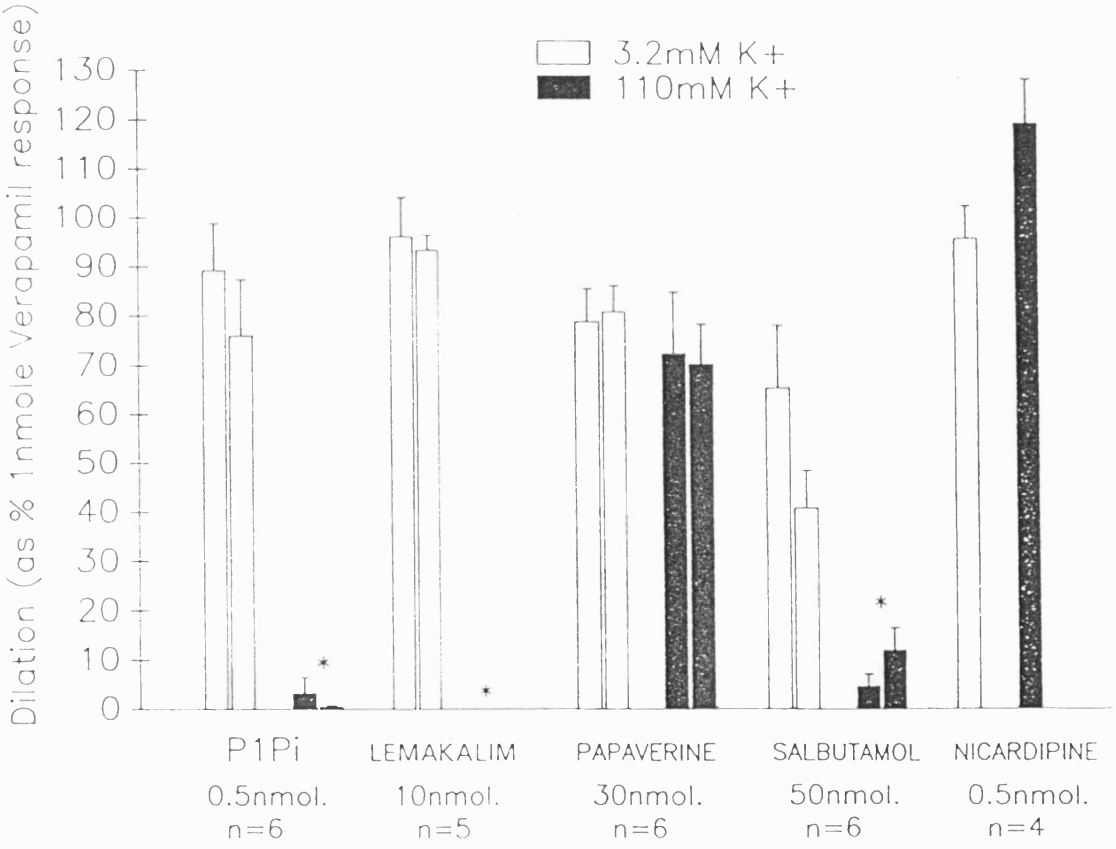
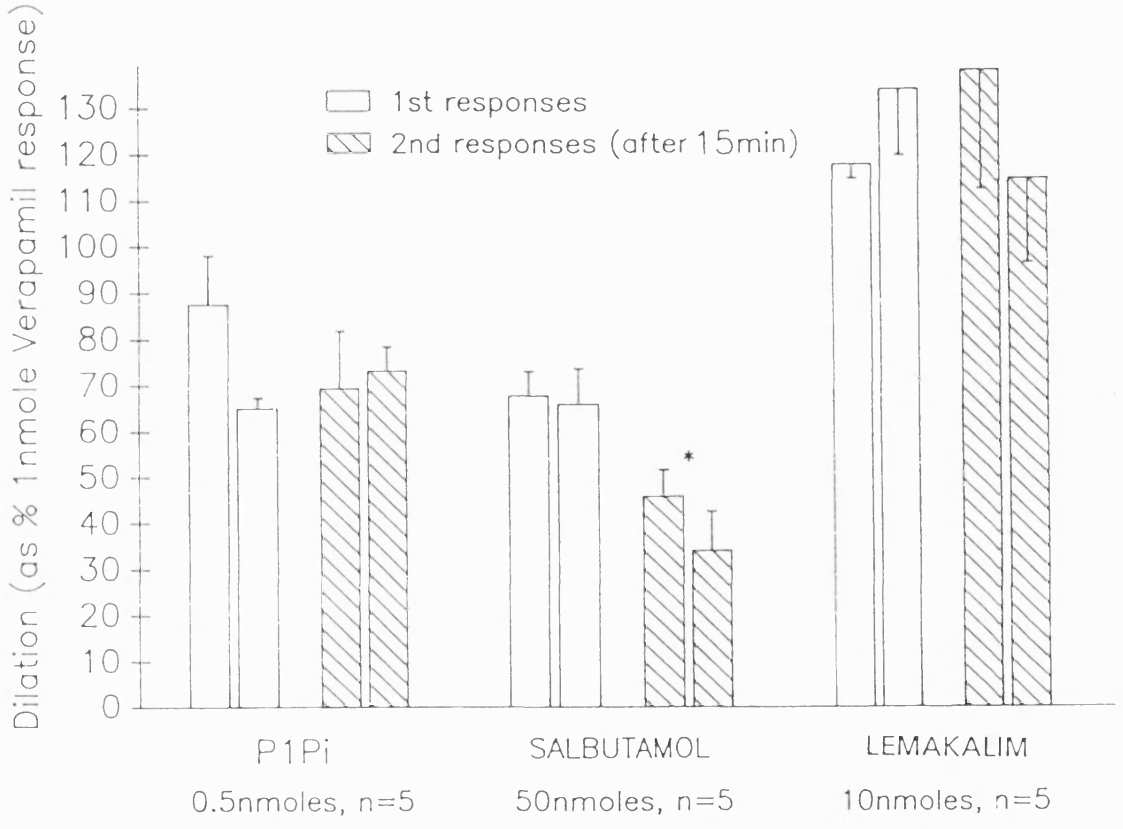


Figure 17

Time-matched control curves to Figure 16 showing the effect of repeated boluses of the coronary dilators P1Pi, salbutamol and lemakalim following 20 minutes time-matched perfusion. Data expressed as % 1nmole verapamil response.

Basal perfusion pressure: 3.2mM $K^+$ 93±8.1mmHg; Following 20 minutes perfusion 127±9.8mmHg n=10, *p<0.05 Student's paired t-test.



### 1.3.2 Effect of 20mM $K^+$ on coronary dilator responses to P1Pi and verapamil.

The effect of 20mM $K^+$ potassium on coronary dilator response to P1Pi was also investigated. This concentration of potassium would produce a depolarisation of the cell, but to a lesser extent than the presence of 110mM $K^+$.

Fitting the Nernst equation (see above):-

$$E_m = 61 .\log_{10} \frac{[20]}{[140]} = -51mV$$

Therefore the presence of 20mM $K^+$ would produce a depolarisation to -51mV, and potassium efflux would still occur following potassium channel activation. A concentration of more than approximately 40mM $K^+$ outside the cell is required to prevent the vasodilator action of potassium channel activators (Hamilton et al., 1986; Chappell et al., 1990).

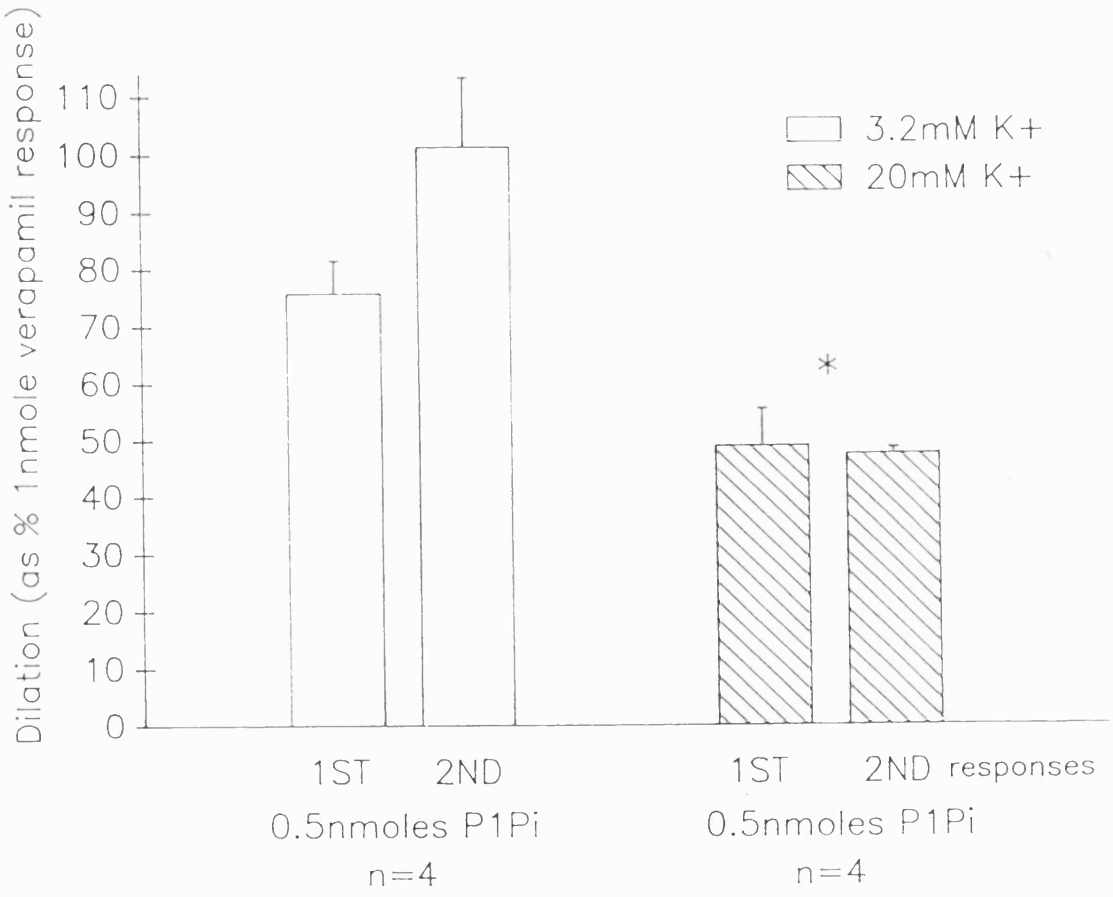
Following perfusion of 20mM $K^+$, a sustained contracture developed in the myocardium in a similar way to that shown in 110mM $K^+$ experiments, accompanied by an increase in perfusion pressure; from $98\pm4.6$mmHg prior to the addition of 20mM $K^+$, to $156\pm6.1$mmHg in the presence of 20mM $K^+$; but again there was no significant difference in the response to verapamil before the addition of 20mM $K^+$ ($42\pm5.6$mmHg, n=4) or in the presence of 20mM $K^+$ ($33\pm3.5$mmHg, n=4). In this case the coronary dilator responses to P1Pi were reduced significantly ($p<0.05$), but not to the same extent as for the experiments using 110mM $K^+$, the responses being reduced by only $43\pm9.7\%$, n=4 in the presence of 20mM $K^+$, and not abolished as shown in 110mM $K^+$ experiments (Figure 18).

Figure 18

Effect of 20mM $K^+$ on the coronary dilator responses to bolus doses of P1Pi in the isolated perfused rat heart. Each dose was repeated to ensure reproducibility. Data expressed as % 1nmole verapamil response.

Basal perfusion pressure: 3.2mM $K^+$ 113±7.5mmHg; 20mM $K^+$ 153±5.2mmHg

n=4, *$p < 0.05$ Student's paired t-test.



**1.4 Effect of tetraethylammonium chloride (TEA) on coronary dilator responses to P1Pi.**

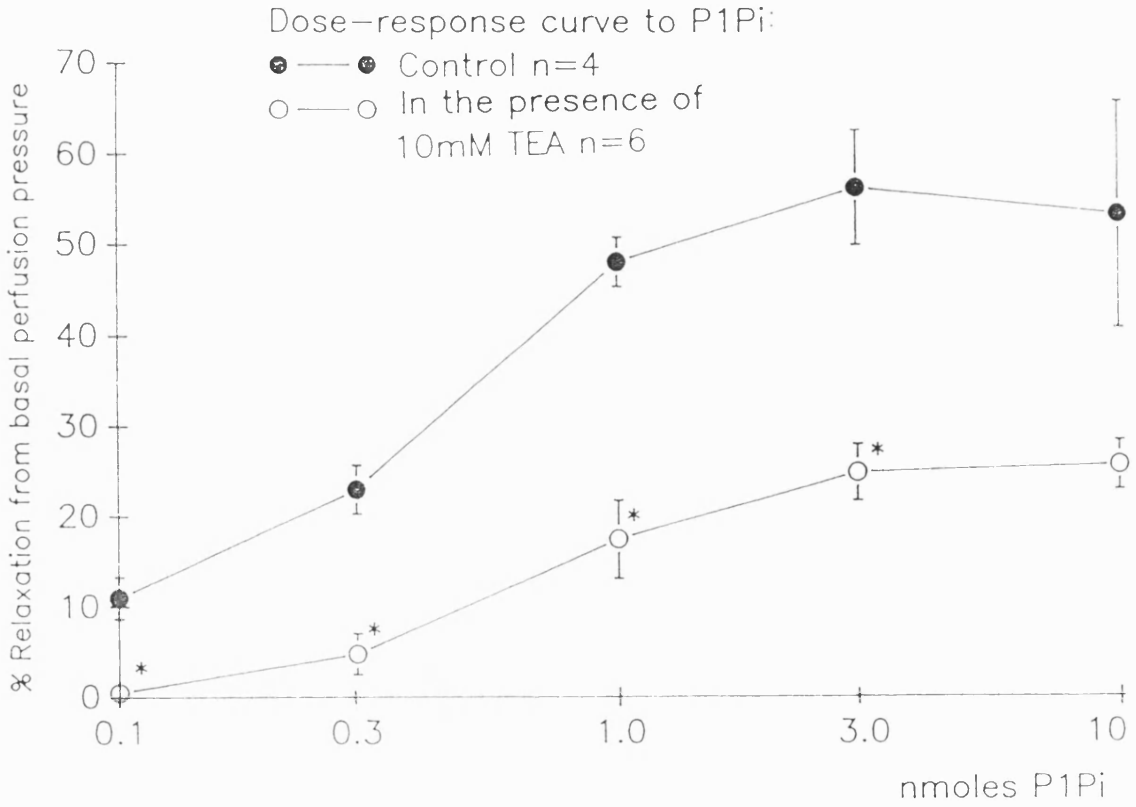
In order to investigate further the effect of P1Pi on $K^+$ channels, the effect of the non-specific $K^+$ channel blocker TEA was investigated.

TEA at a concentration of 10mM produced an increase in perfusion pressure, from $81 \pm 7.9$mmHg in the absence of TEA, to $112 \pm 15.8$mmHg in the presence of 10mM TEA (n=6). The same concentration of TEA produced a fall in heart rate of $57 \pm 14$bpm from a basal heart rate of $286 \pm 6$bpm, n=6. There was no significant effect on developed tension. It can be seen from Figure 19 that 10mM TEA did not abolish coronary dilator responses to P1Pi. However there was a significant depression of the coronary dilator responses to P1Pi, although the dose of P1Pi producing the maximum dilator response remained the same. The coronary dilator response to 1nmole verapamil, used as an internal control, was not significantly altered before the addition of TEA: $35 \pm 3.3\%$ relaxation from basal perfusion pressure, or in the presence of TEA: $37 \pm 5.0\%$ relaxation, n=6.

Figure 19

Dose-response curve showing coronary dilator responses to P1Pi in the absence and presence 10mM tetraethylammonium chloride (TEA). Data expressed as % relaxation from basal perfusion pressure.

Basal (elevated) perfusion pressure: Control 114±3.1mmHg n=4; 10mM TEA 112±16mmHg n=6, *$p<0.05$ Student's unpaired t-test.



## 1.5 Effect of P1Pi on the perfusion pressure, developed tension and heart rate responses to BAY K 8644, noradrenaline, $PGF_{2\alpha}$, methoxamine and caffeine .

These experiments were performed in order to investigate the interaction of P1Pi with various vasoconstrictor compounds, in order to further elucidate the mechanism(s) of coronary dilation of P1Pi.

### 1.5.1 Effect of P1Pi on basal parameters.

At a concentration of 1nM, P1Pi had no effect on any of the basal parameters measured. Increasing the concentration of P1Pi to 10nM had no effect on basal developed tension and heart rate, but coronary dilation did occur, as indicated by a fall in perfusion pressure of $19\pm2.8$mmHg to a mean value of $68\pm4.1$mmHg (n=24).

In experiments where the concentration of P1Pi was increased to 100nM, P1Pi caused an initial fall in perfusion pressure of $12\pm1.3$mmHg (n=9), which was not associated with any change in developed tension or heart rate. Increasing the concentration of P1Pi to 1$\mu$M did not produce a further fall in perfusion pressure. This concentration also had no effect on developed tension or heart rate (n=5).

### 1.5.2 Effect of P1Pi on responses to BAY K 8644.

In these experiments, the basal perfusion pressure, heart rate and developed tension were $69\pm8.2$mmHg, $271\pm23$bpm, and $6.9\pm0.7$g respectively.

Bolus doses of BAY K 8644 (10-100pmol.) produced well defined, transient, dose-dependent increases in perfusion pressure with small, inconsistent increases in

developed tension. Heart rate was not affected. Doses above 100pmol were not used in control curves as perfusion pressures would have exceeded 200mmHg.

P1Pi (10nM - 1$\mu$M) produced a concentration dependent inhibition of the coronary constrictor action of BAY K 8644 (Figures. 20 & 21). There was no significant shift of the dose-response curve in time-matched control experiments (data not shown).

Figure 20

Trace showing the effects of P1Pi on the perfusion pressure (PP), developed tension (DT) and heart rate (HR) responses to bolus doses of BAY K 8644 in the isolated perfused rat heart.

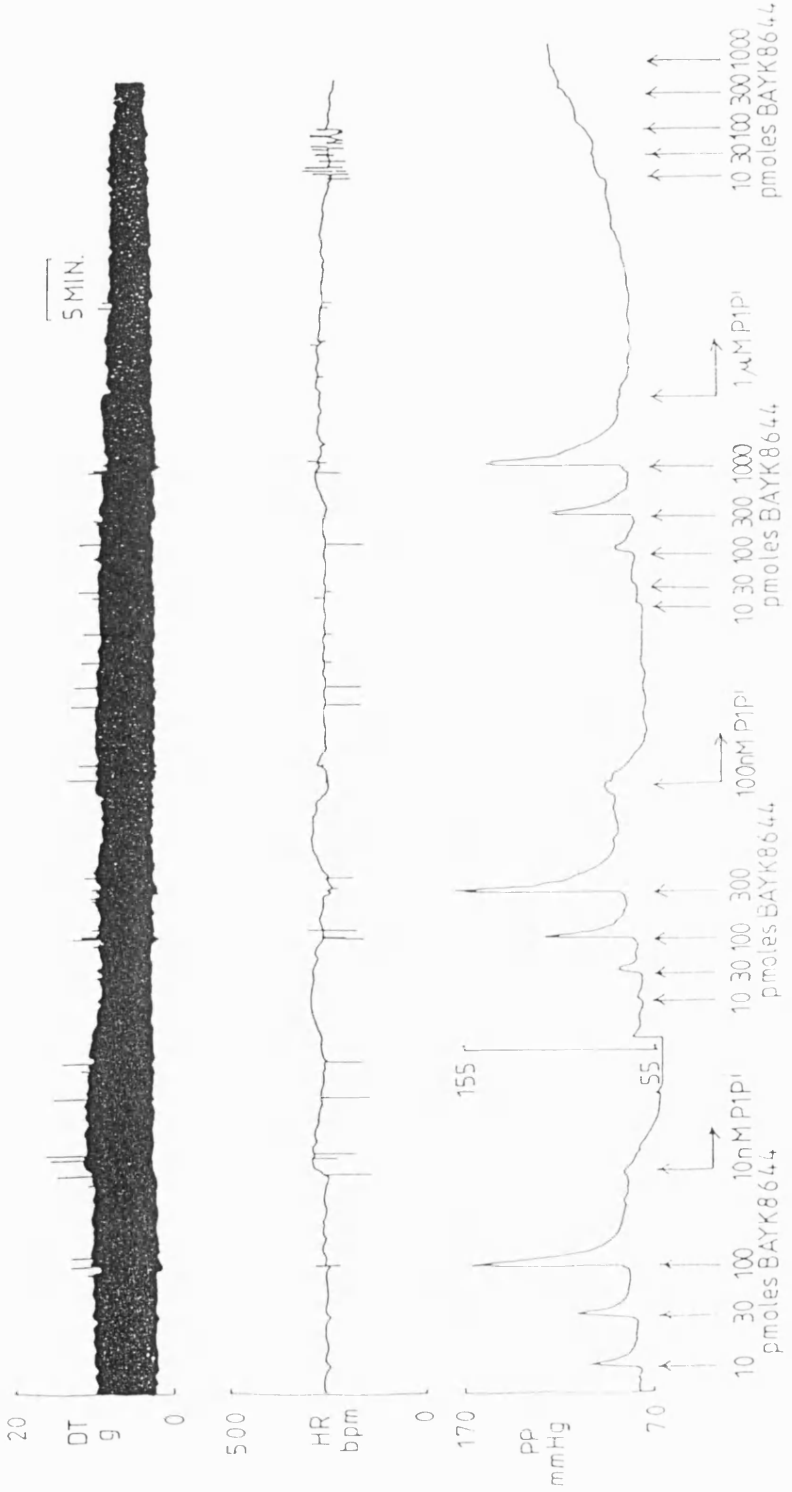
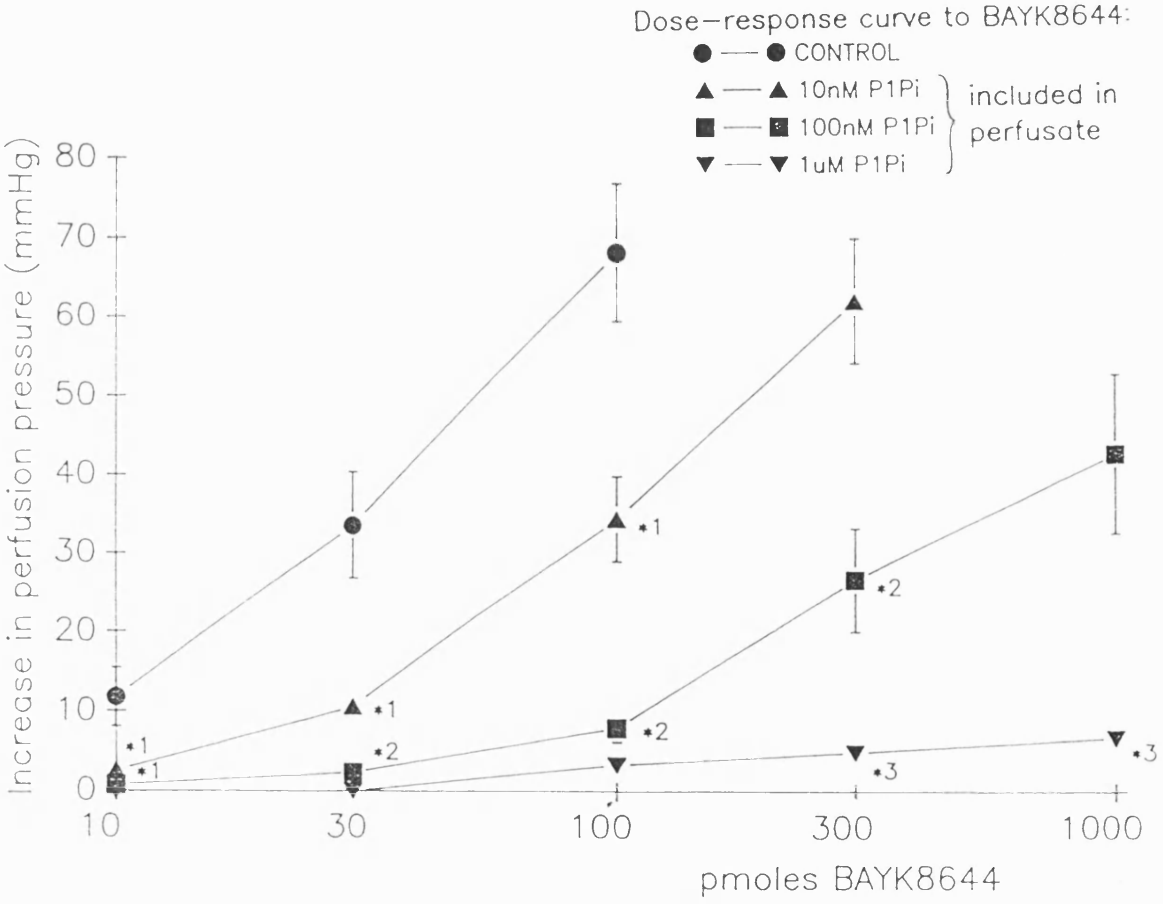


Figure 21

Effect of P1Pi on the coronary constrictor responses to bolus doses of BAY K 8644 in the isolated perfused rat heart. Data expressed as mmHg increase in perfusion pressure.

Basal perfusion pressure: Control 69±8.2mmHg; 10nM P1Pi 57±1.4mmHg; 100nM P1Pi 54±2.0mmHg; 1μM P1Pi 61±4.3mmHg n=5, *1 $p<0.05$ compared with Control, *2 $p<0.05$ compared with 10nM P1Pi, *3 $p<0.05$ compared with 100nM P1Pi, 2 way ANOVA.



### 1.5.3 Effect of P1Pi on responses to noradrenaline.

The initial aim of these experiments was to investigate the effect of P1Pi on the coronary constrictor action of noradrenaline. However, it can be seen from Figure 22 that noradrenaline produced only a very small degree of coronary constriction, preceding a large coronary dilator response at higher doses. This was accompanied by dose-related positive inotropic and chronotropic effects (Figure 22). The positive inotropic effect was biphasic, consisting of an initial, transient inotropic effect, followed by a later, more prolonged inotropic response. Following perfusion with P1Pi (1nM and 10nM) the second phase of the positive inotropic response to noradrenaline was significantly depressed in a concentration dependent manner (Figure 22 & 23). In contrast, the chronotropic action of noradrenaline was not affected by P1Pi (Figure 22 & 24). At 10nM, P1Pi tended to reduce the coronary dilator action of noradrenaline, but this effect was not significant (Figure 25).

Figure 22

Trace showing the effect of P1Pi on perfusion pressure, developed tension and heart rate responses to noradrenaline in the isolated perfused rat heart.

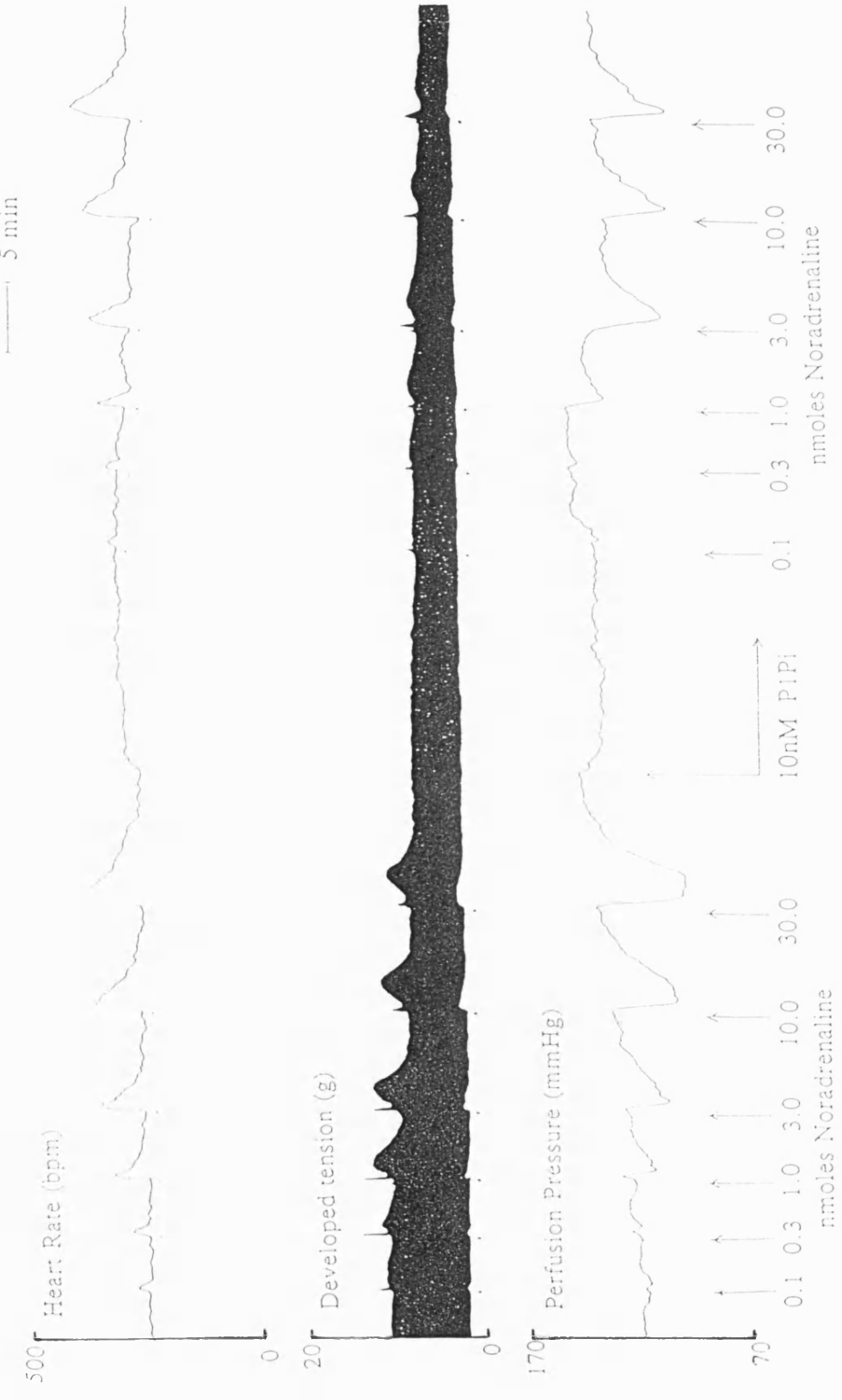


Figure 23

Effect of P1Pi and atenolol on the positive inotropic response to noradrenaline in the isolated, perfused rat heart. Data expressed as a % of the basal developed tension. Basal developed tension: Time matched control 5.4±0.4g n=9; 1nM P1Pi 4.2±0.7g n=6; 10nM P1Pi 5.2±0.5g n=6; 1μM Atenolol 5.6±1.1g n=5, *p<0.05, compared with time-matched controls, 1 way ANOVA.

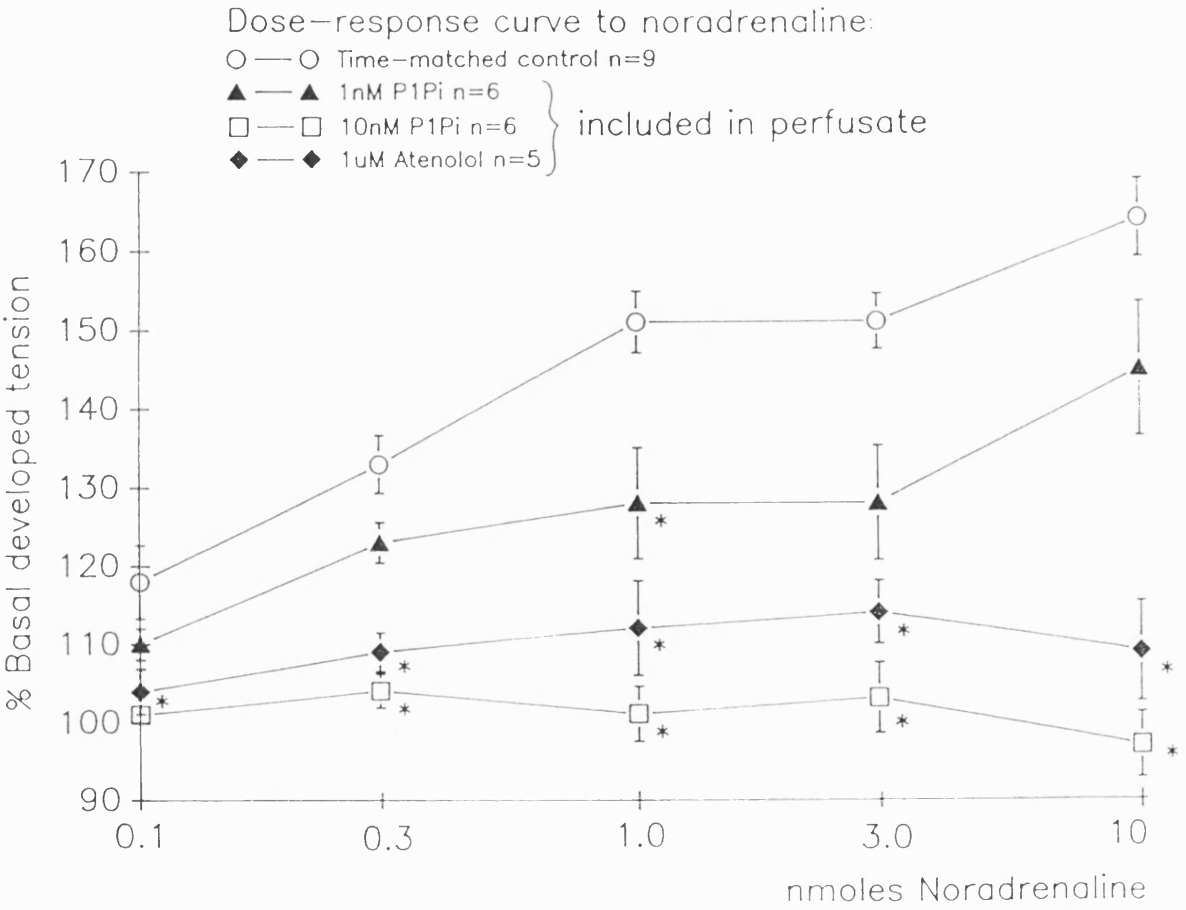


Figure 24

Effect of P1Pi and atenolol on the positive chronotropic response to noradrenaline in the isolated, perfused rat heart. Data expressed as bpm above basal heart rate. Basal heart rate: Time matched control 277±10bpm n=9; 1nM P1Pi 297±12bpm n=6; 10nM P1Pi 260±19bpm n=6; 1μM Atenolol 277±4bpm n=5, *p<0.05, compared with time-matched controls, 1 way ANOVA.

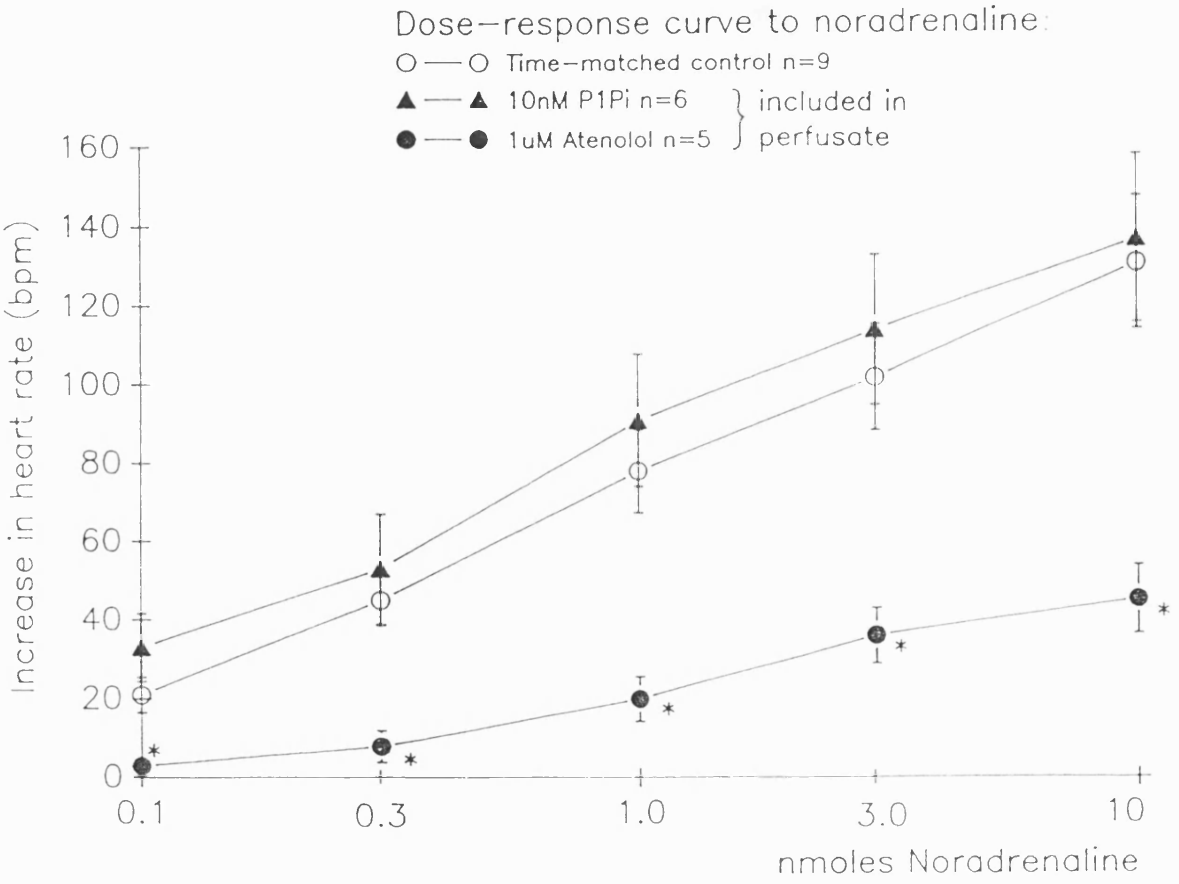
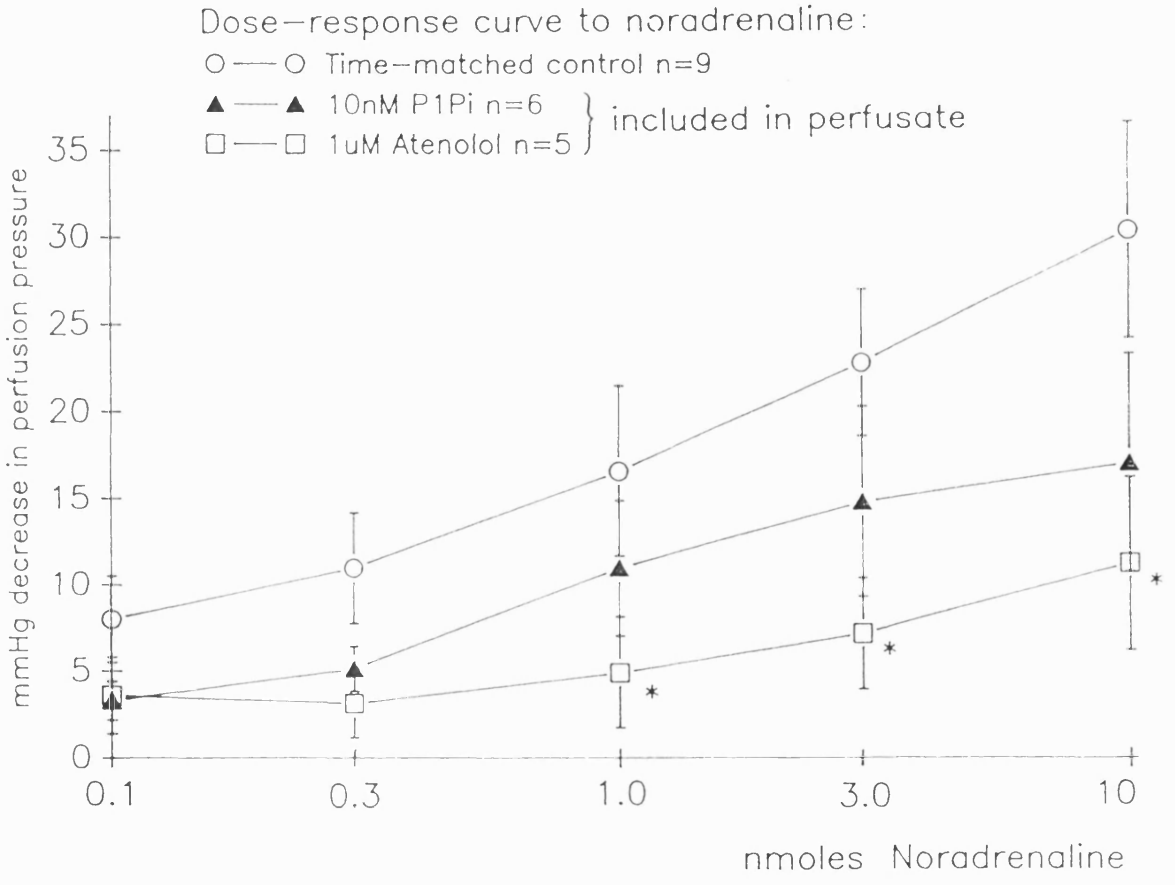


Figure 25

Effect of P1Pi and atenolol on the coronary dilator responses to noradrenaline in the isolated perfused rat heart. Data expressed as mmHg decrease in perfusion pressure. Basal perfusion pressures: Time matched control 87±8.0mmHg n=9; 10nM P1Pi 90±15mmHg n=6; 1μM Atenolol 102±8.0mmHg n=5, *$p<0.05$, compared with time matched controls, one way ANOVA.



### 1.5.4 Effect of atenolol on responses to noradrenaline.

In order to investigate why P1Pi had a selective effect on the inotropic and not the chronotropic effect of noradrenaline, the effects of P1Pi were compared to those of the $ß_1$-adrenoceptor antagonist atenolol. For example the lack of any effect on heart rate may be due to a poor perfusion of the SA node in this preparation. If adequate perfusion of the SA node were present, the $ß_1$-adrenoceptor antagonist atenolol should antagonise both the positive inotropic and the positive chronotropic effect of noradrenaline.

In contrast to P1Pi, atenolol at 1$\mu$M significantly reduced both the inotropic and chronotropic actions of noradrenaline (Figures 23 & 24). At the concentration used, atenolol also significantly reduced the coronary dilator action of noradrenaline. This inhibition was greater than that shown with 10nM P1Pi (Figure 25).

### 1.5.5 Effect of palmitoyl carnitine on responses to noradrenaline.

In view of the results obtained with P1Pi, further experiments were carried out in order to investigate whether the parent compound, palmitoyl carnitine, was able to produce the same effect.

Palmitoyl carnitine at 10nM produced an increase in perfusion pressure from $85 \pm 11.5$mmHg to $115 \pm 16.5$mmHg (n=6). It produced no significant effect on developed tension or heart rate. Palmitoyl carnitine at 10nM had no significant effect on the positive inotropic responses to noradrenaline, measured as a % increase from basal developed tension (Figure 26).

The positive chronotropic responses were also not significantly affected by palmitoyl carnitine; in time matched control curves 3nmoles noradrenaline produced an increase

in heart rate of 102±14bpm, n=9; whereas in the presence of 10nM palmitoyl carnitine the increase was 92±14bpm, n=5. The coronary dilator response to 3nmoles noradrenaline was also not significantly affected; at 3nmoles a coronary dilator response of 23±4.2mmHg, n=9, was produced in time matched control curves, and 22±6.1mmHg, n=6, in the presence of 10nM palmitoyl carnitine.

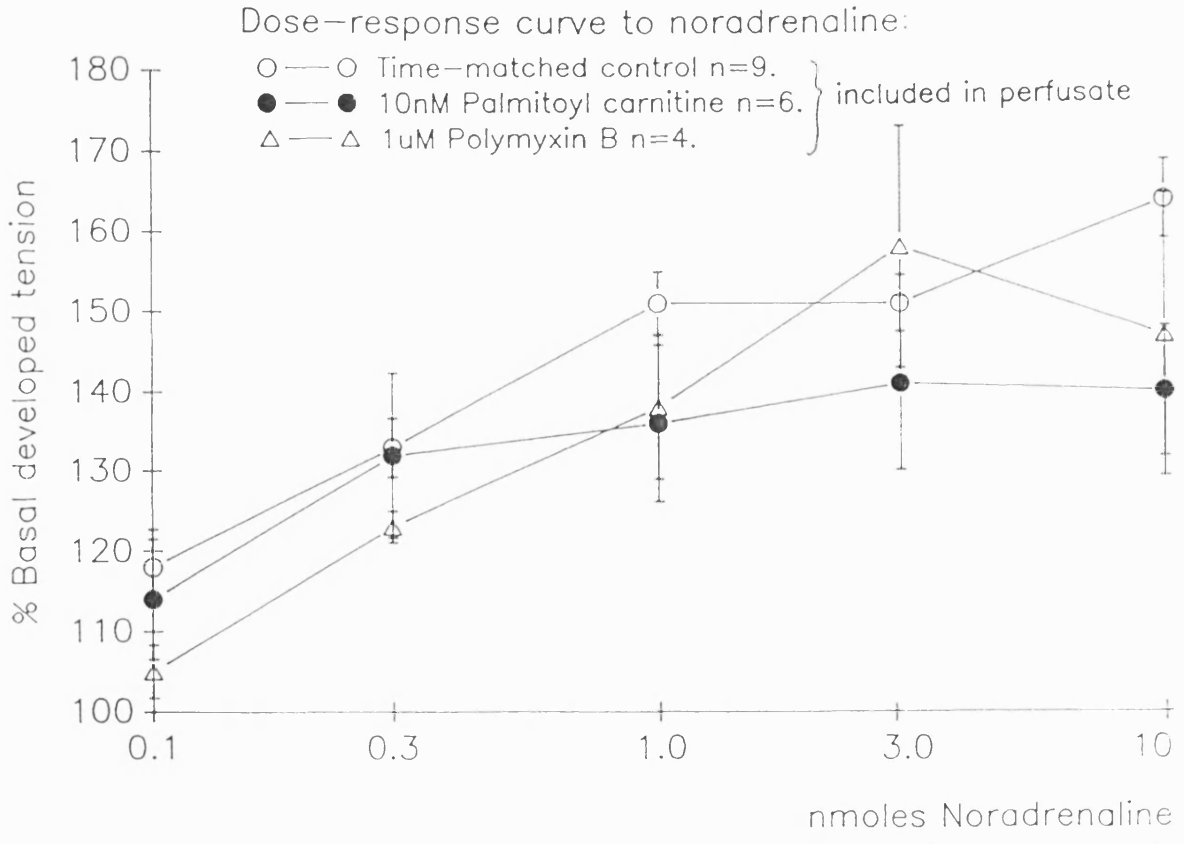
### 1.5.6 Effect of polmyxin B on responses to noradrenaline.

The fact that P1Pi inhibited the positive inotropic effect of noradrenaline, while the parent compound palmitoyl carnitine did not may be related to the fact that P1Pi is a positively charged amphiphile, while palmitoyl carnitine is a zwitterion. In order to investigate this possibility further, the effect of another cationic amphiphile, polymyxin B, on noradrenaline responses was compared with the actions of P1Pi and palmitoyl carnitine. Polymyxin B at 1$\mu$M slightly increased perfusion pressure, 79±13.9mmHg prior to the addition of polymyxin B; 102±17.2mmHg in the presence of polymyxin B, but this increase was not significant. Basal heart rate and developed tension were also not significantly affected. A concentration of 1$\mu$M polymyxin B also had no significant effect on the positive inotropic response to noradrenaline (Figure 26). In addition, positive chronotropic and coronary dilator responses induced by noradrenaline were not affected. At a submaximal dose of noradrenaline (3nmoles) the increase in heart rate was 102±14bpm, n=9, in the absence and 89±27bpm, n=4, in the presence of 1$\mu$M polymyxin B. The same dose (3nmoles) of noradrenaline produced a coronary dilation of 23±4.2mmHg, n=9, in the absence and 28±9.6mmHg, n=4, in the presence of 1$\mu$M polymyxin B.

Figure 26

The effect of palmitoyl carnitine and polymyxin B on the positive inotropic response to noradrenaline in the isolated perfused rat heart. Data expressed as % of the basal developed tension.

Basal developed tension: Time matched control 5.4±0.4g n=9; 10nM palmitoyl carnitine 3.6±0.3g n=6; polymyxin B 4.6±0.4g n=4, *$p<0.05$, compared with time-matched controls, one way ANOVA.



### 1.5.7 Effect of P1Pi on responses to $PGF_{2\alpha}$.

In order to investigate whether P1Pi could suppress the responses to vasoconstrictors other than BAY K 8644, the effect of P1Pi on $PGF_{2\alpha}$, shown to be a potent constrictor in coronary smooth muscle (Nakajima & Ueda 1990; Balwierczak 1991) was investigated.

$PGF_{2\alpha}$ produced a very small, variable coronary constrictor effect. However, it also produced a monophasic, dose-related positive inotropic effect with no concomitant effect on heart rate (Figure 27). A concentration of 10nM P1Pi significantly reduced the positive inotropic effect of $PGF_{2\alpha}$ (Figure 28). Coronary constrictor responses were not large enough to be measured accurately.

Figure 27

Trace showing the effect of P1Pi on the developed tension (DT), heart rate (HR) and perfusion pressure (PP) responses to bolus doses of $PGF_{2\alpha}$ in the isolated perfused rat heart.

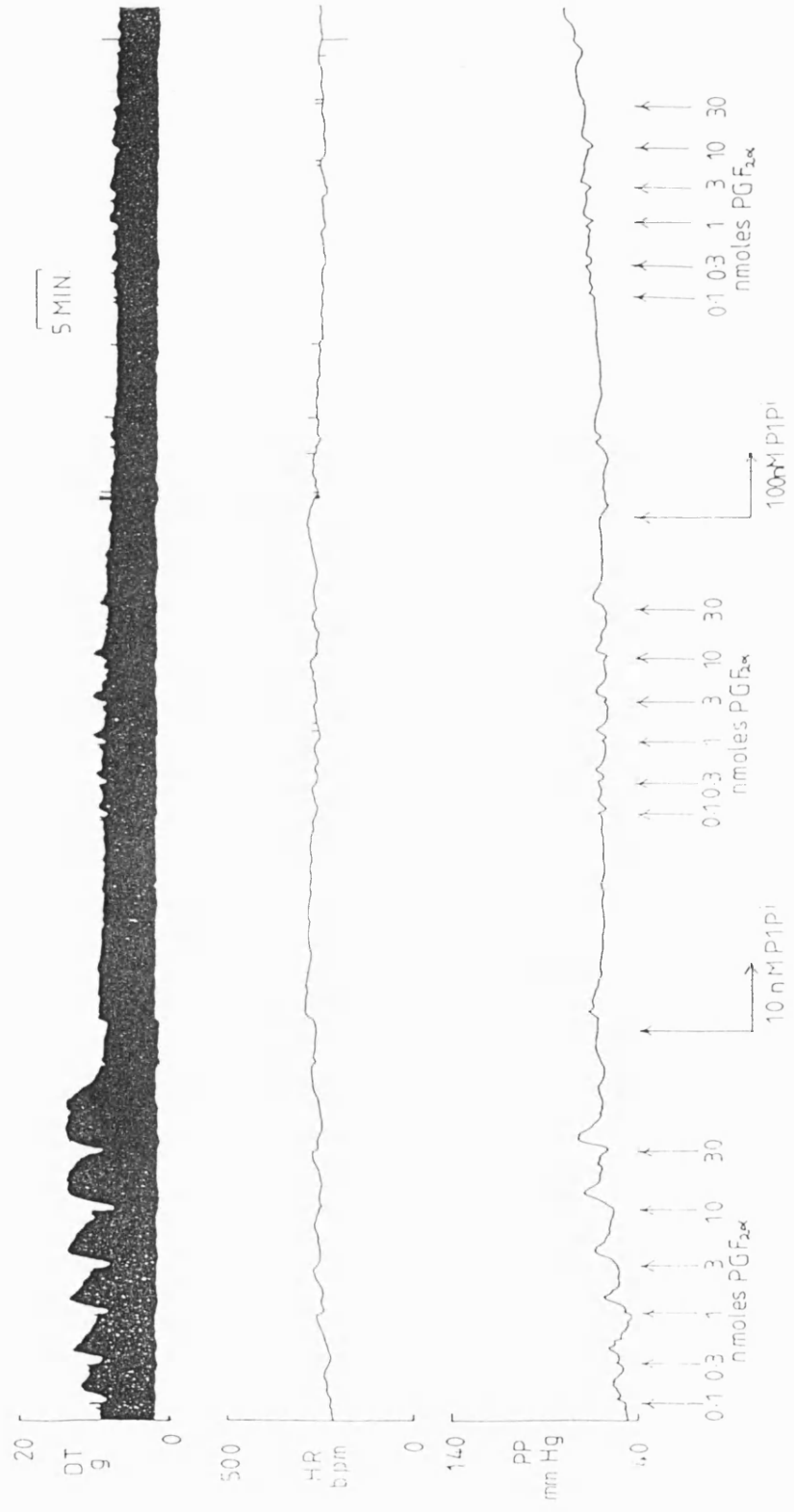
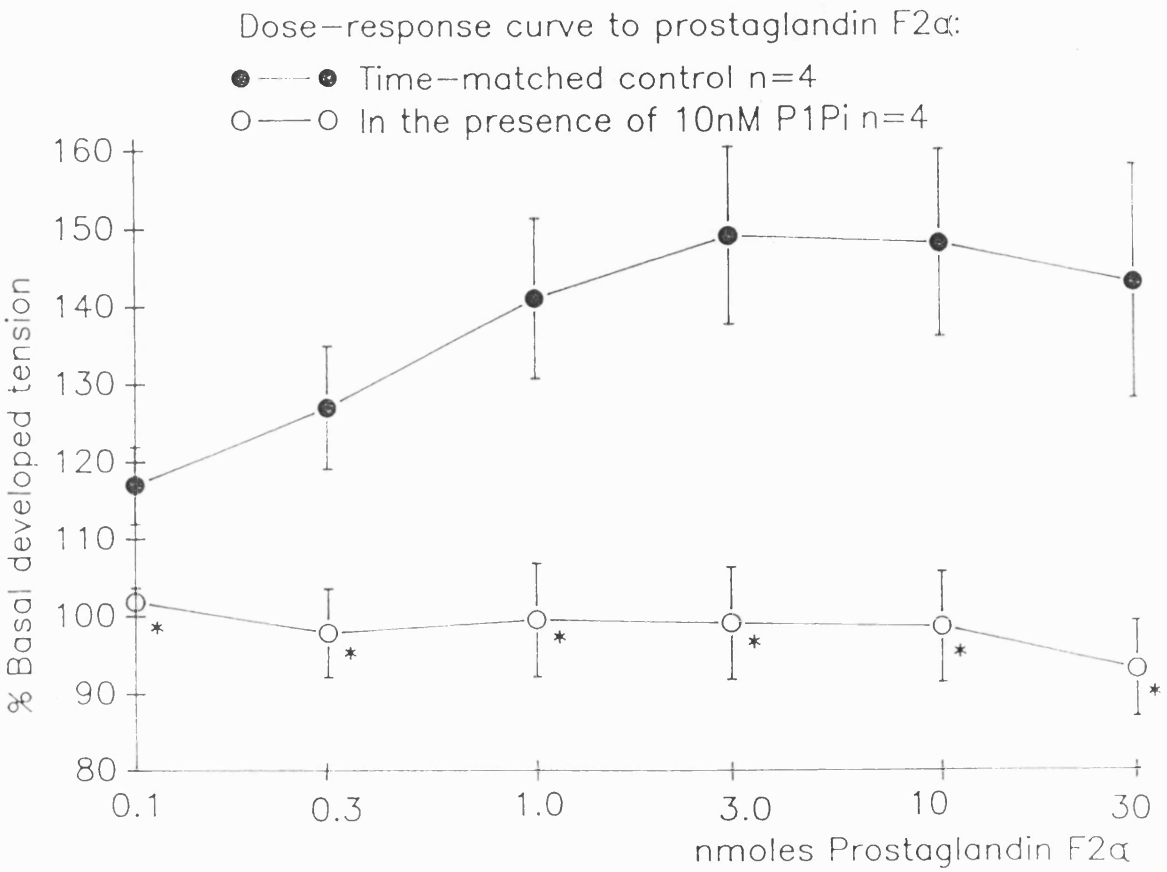


Figure 28

The effect of P1Pi on the positive inotropic responses to $PGF_{2\alpha}$ in the isolated perfused rat heart, expressed as % of the basal developed tension.

Basal developed tension: Time-matched control 6.8±0.7g n=4; 10nM P1Pi 7.4±0.8g n=4, *$p < 0.05$ Student's unpaired t-test.



### 1.5.8 Effect of P1Pi on responses to methoxamine.

In order to investigate whether the ability of P1Pi to suppress agonist-induced positive inotropic responses could be applied to other agonists, as well as noradrenaline and $PGF_{2\alpha}$, the effect of P1Pi on responses to the α-adrenoceptor agonist methoxamine was investigated.

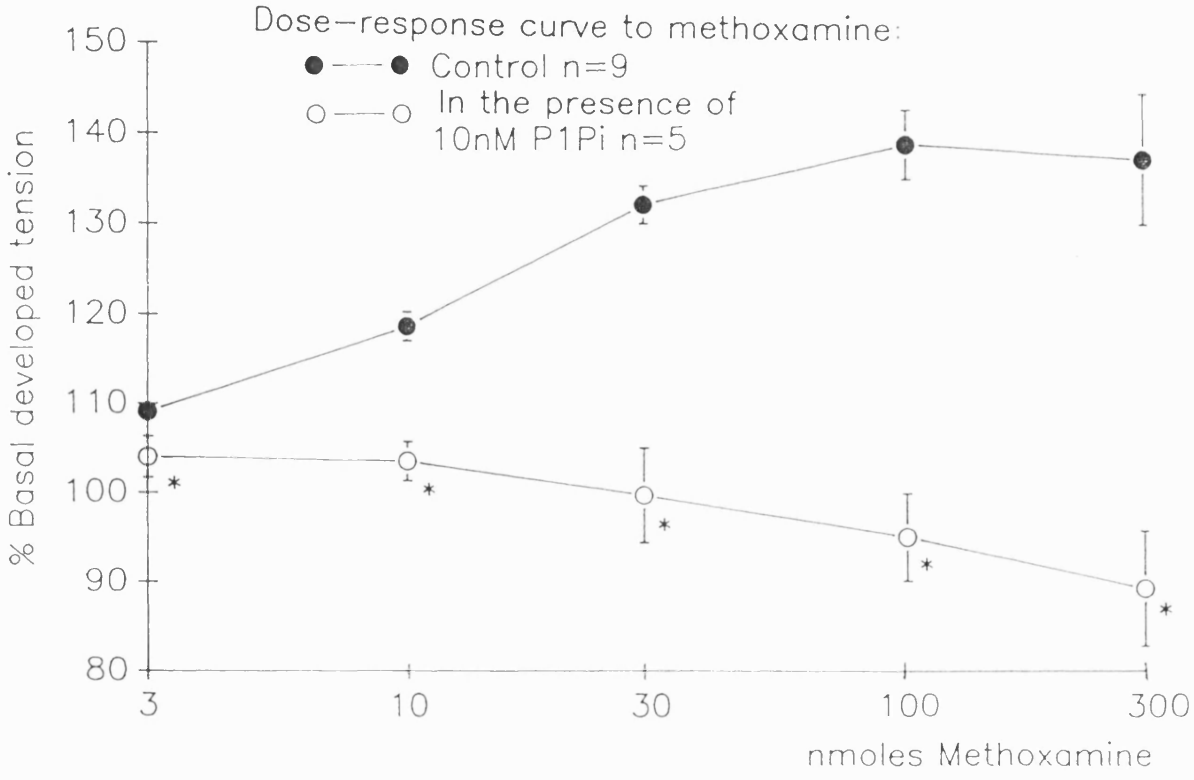
Positive inotropic responses to methoxamine were found to be subject to tachyphylaxis on repeating the dose-response curve. Therefore in these experiments, 1st dose-response curves were carried out in the presence of P1Pi for test experiments, and control curves in separate preparations. Methoxamine produced a dose-related positive inotropic effect which was monophasic with no significant effect on heart rate, and a variable effect on perfusion pressure.

At 10nM, P1Pi significantly reduced the methoxamine-induced positive inotropic response, as shown in Figure 29. Coronary constrictor responses were not measured as these showed a high degree of variability between preparations.

Figure 29

Effect of P1Pi on the positive inotropic response to methoxamine in the isolated perfused rat heart. Data expressed as % of the basal developed tension. First dose-response curves shown, in the presence or absence of P1Pi.

Basal developed tension: Time-matched control 6.1±0.7g n=9; 10nM P1Pi 7.1±1.3g n=5, *p < 0.05 Student's unpaired t-test.



### 1.5.9 Effect of P1Pi on responses to caffeine.

The action of P1Pi to suppress agonist-induced positive inotropic responses could involve an inhibition of calcium influx or calcium release from the sarcoplasmic reticulum. However, the inhibition of BAY K 8644-induced positive inotropic responses appears to suggest an inhibition of calcium influx thorough L-type calcium channels in vascular smooth muscle. In order to investigate whether P1Pi could influence calcium release from the sarcoplasmic reticulum, the effect of P1Pi on caffeine responses was investigated.

Figure 30 shows the effect of caffeine on the three parameters measured. Caffeine (0.3-30$\mu$moles) produced a small, transient coronary constrictor response, associated in some cases with a transient positive inotropic effect with a duration of only 2-3 beats, followed by a longer lasting coronary dilator effect. Heart rate was unaffected. P1Pi at both 10nM and 100nM had no significant effect on the coronary constrictor responses to caffeine (Figure 31). The coronary dilator response was significantly ($p < 0.05$) attenuated in the presence of 10 or 100nM P1Pi. In the absence of P1Pi, a fall of $18.0 \pm 5.8$mmHg perfusion pressure was produced with 30$\mu$moles caffeine, in the presence of 10nM P1Pi this was reduced to $3.8 \pm 1.8$mmHg, and in the presence of 100nM P1Pi the coronary dilator response was abolished.

Positive inotropic responses were not measured, due to the variability between preparations.

Figure 30

Trace showing the effect of P1Pi on coronary perfusion pressure, developed tension and heart rate responses to bolus doses of caffeine in the isolated perfused rat heart.

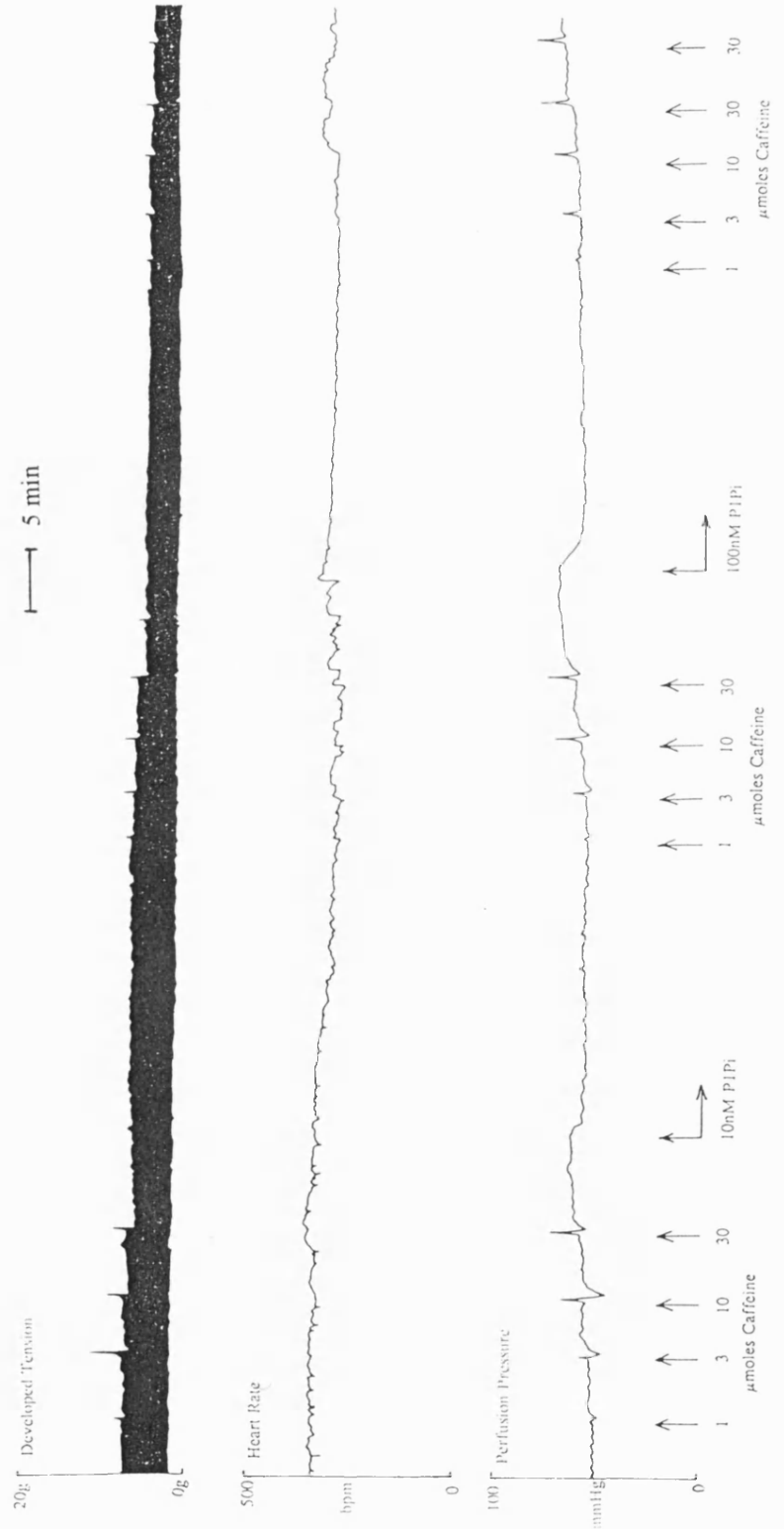
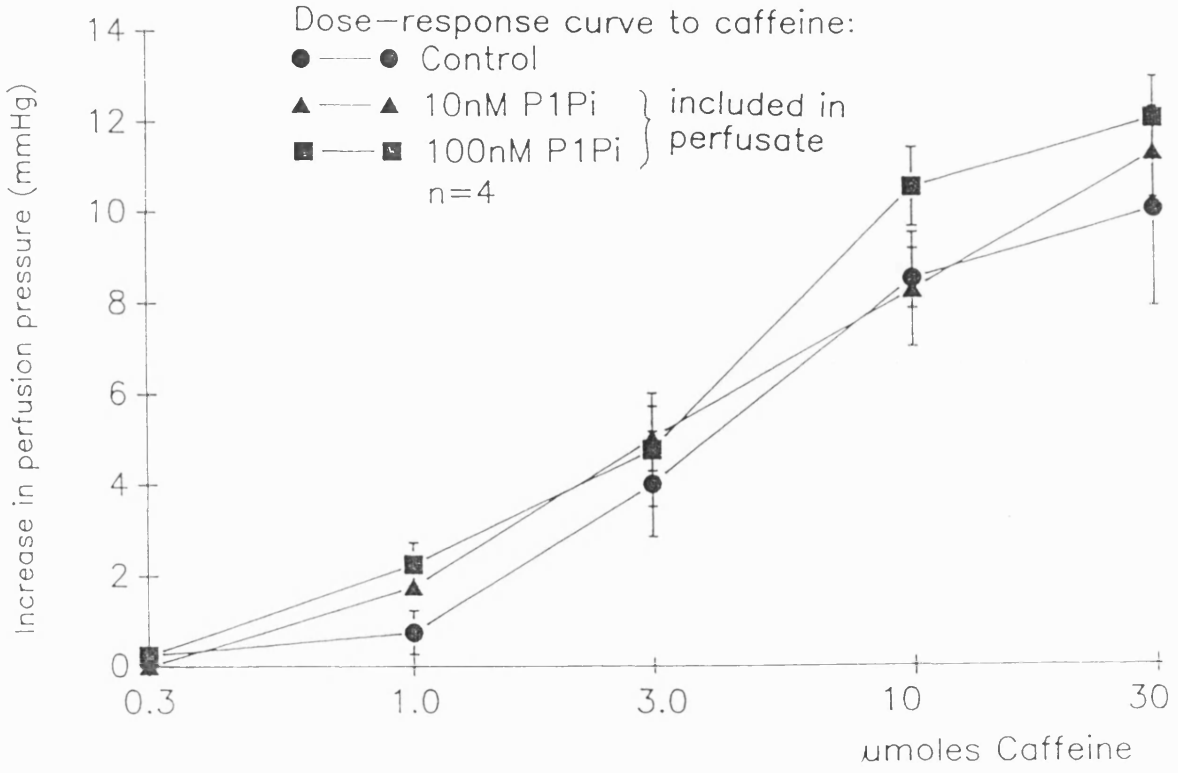


Figure 31

Effect of P1Pi on the coronary constrictor responses to bolus doses of caffeine in the isolated, perfused rat heart. Data expressed as mmHg increase from basal perfusion pressure.

Basal perfusion pressure: Control 71±7.4mmHg, 10nM P1Pi 69±6.4mmHg, 100nM P1Pi 72±5.3mmHg n=4.



**1.6 Effect of noradrenaline following washout of P1Pi (reversibility experiments).**

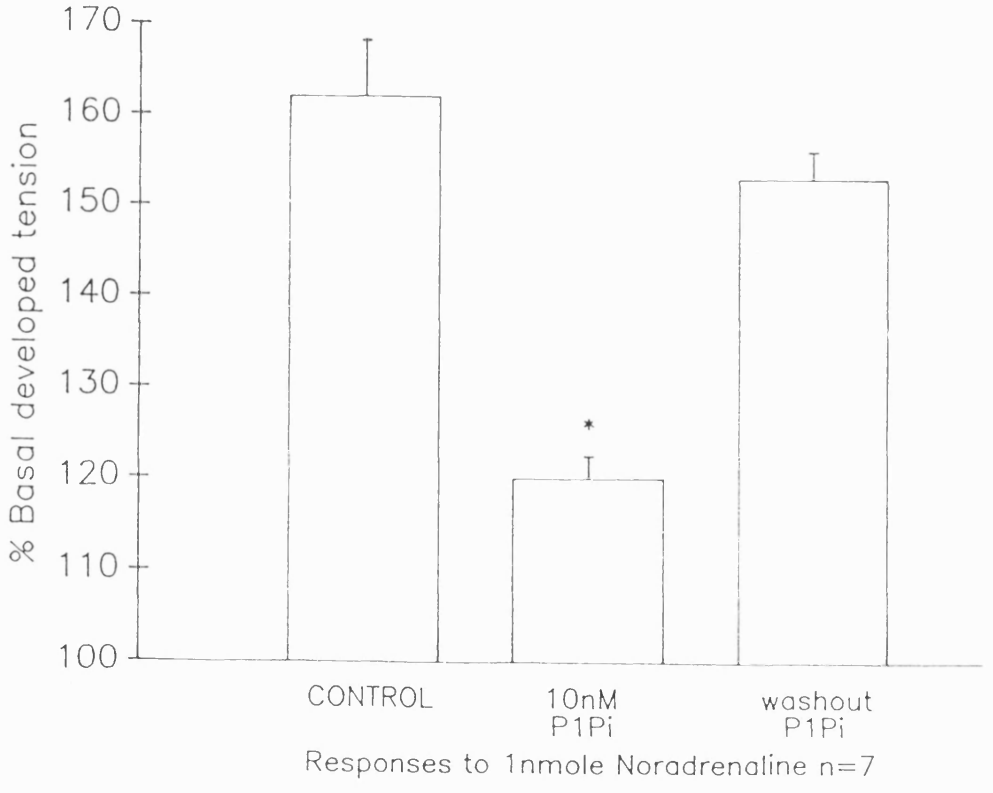
These experiments were carried out to investigate the reversibility of the action of P1Pi, to suppress the positive inotropic response to noradrenaline.

It can be seen from Figure 32 that the inhibitory effect of 10nM P1Pi on the positive inotropic response to 1nmole noradrenaline was completely reversed following 40 minutes washout of P1Pi.

Figure 32

Effect of a 1nmole bolus of noradrenaline on developed tension before perfusion of P1Pi, in the presence of P1Pi and following 40 minutes washout of P1Pi. Data expressed as % of the basal developed tension prior to each 1nmole bolus of noradrenaline administered.

Basal developed tension: Prior to 1st 1nmole dose noradrenaline (control) 8.4±0.7g; In presence of 10nM P1Pi 8.5±0.8g; following 40 minutes washout (w/o) P1Pi 5.7±0.7g n=7, *p<0.05 Student's paired t-test, compared with initial 1nmole noradrenaline control response.



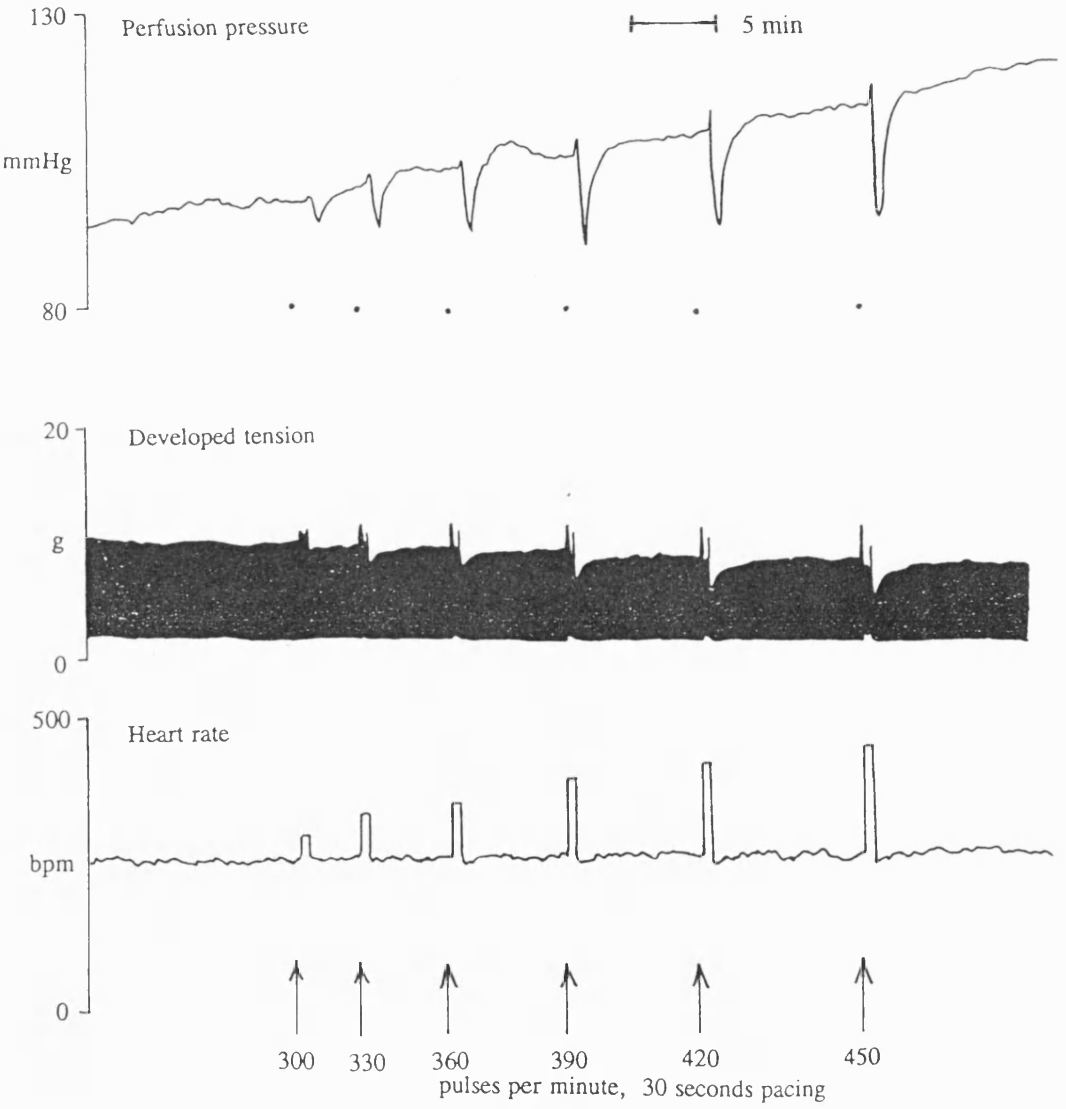
**1.7 Effect of increasing the heart rate by electrical pacing in the isolated perfused heart on developed tension and perfusion pressure.**

In order to investigate the influence of an increase in heart rate on the other parameters measured, to help explain the responses obtained in response to noradrenaline in previous experiments, hearts were paced at intervals at increasing frequencies.

Figure 33 is a representative trace showing the effect of heart rate on developed tension and perfusion pressure. There was a frequency-dependent coronary dilator effect which increased as the stimulation frequency was increased. There was also a frequency dependent immediate positive inotropic effect, followed by a delayed, frequency dependent depression of contractility in response to an increase in heart rate. This pattern was observed in each experiment performed (n=6).

Figure 33

Trace showing the effects of electrical pacing for 30 seconds on developed tension and perfusion pressure in the isolated, perfused rat heart.



### 1.8.1 Effect of sequentially lowering the sodium concentration in the perfusate on developed tension.

Experiments using low sodium perfusion were performed in order to investigate the effect of P1Pi on positive inotropic and coronary constrictor responses elicited by modulation of $Na^{+}$-$Ca^{2+}$ exchange.

The trace in Figure 34 shows that sequentially lowering the concentration of sodium in the perfusate, from 118 to 89 to 59 to 44mM NaCl, produced a sequential increase in developed tension, a depression of heart rate and complex effects on perfusion pressure. The effect of sodium concentration on developed tension is shown in Figure 35.

Figure 34

Representative trace showing the effect of progressively lowering the sodium chloride concentration in the perfusate on developed tension, perfusion pressure and heart rate in the isolated perfused rat heart.

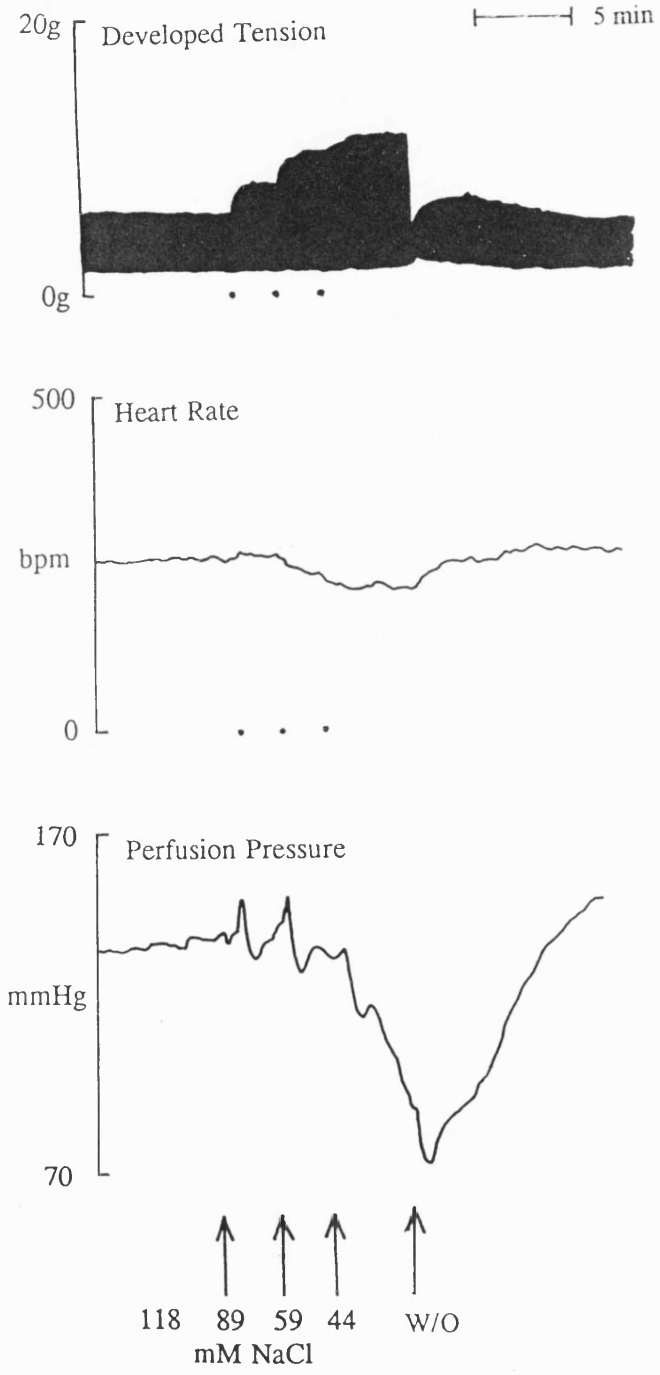
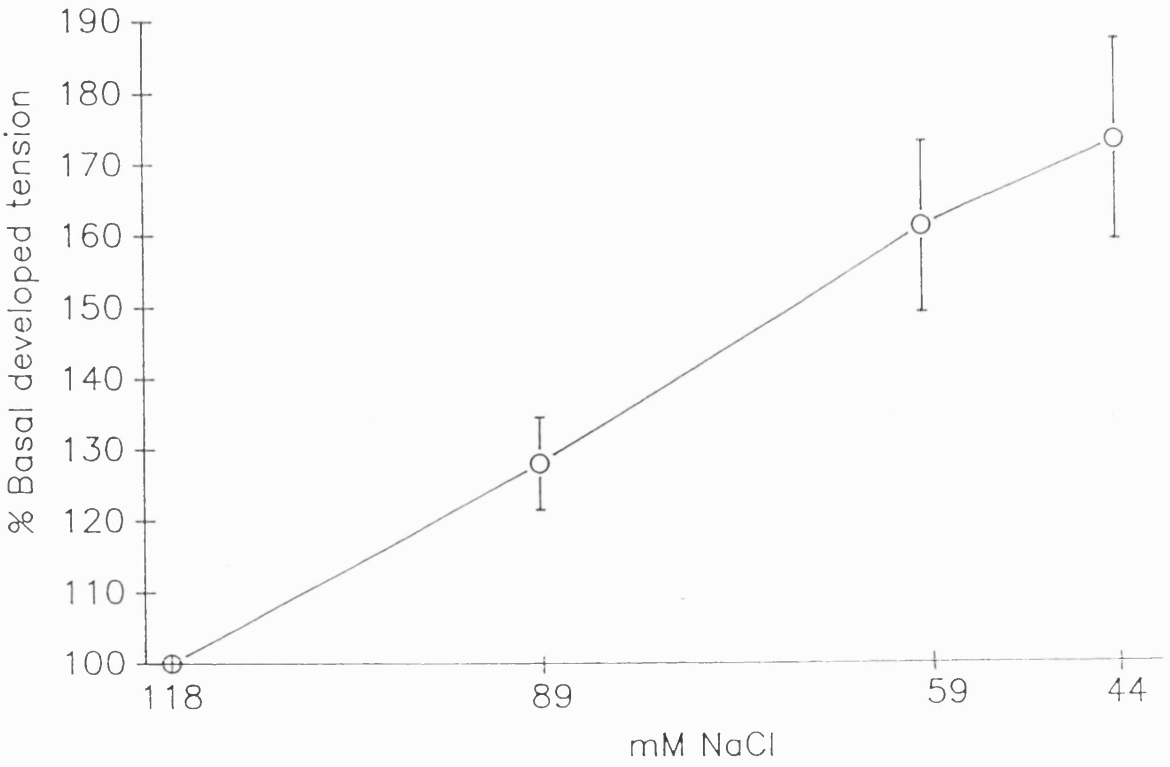


Figure 35

Effect of progressively lowering the sodium chloride concentration in the perfusate on developed tension in the isolated, perfused rat heart. Data expressed as % of the basal developed tension.

Basal developed tension 8.0±0.8g n=4.

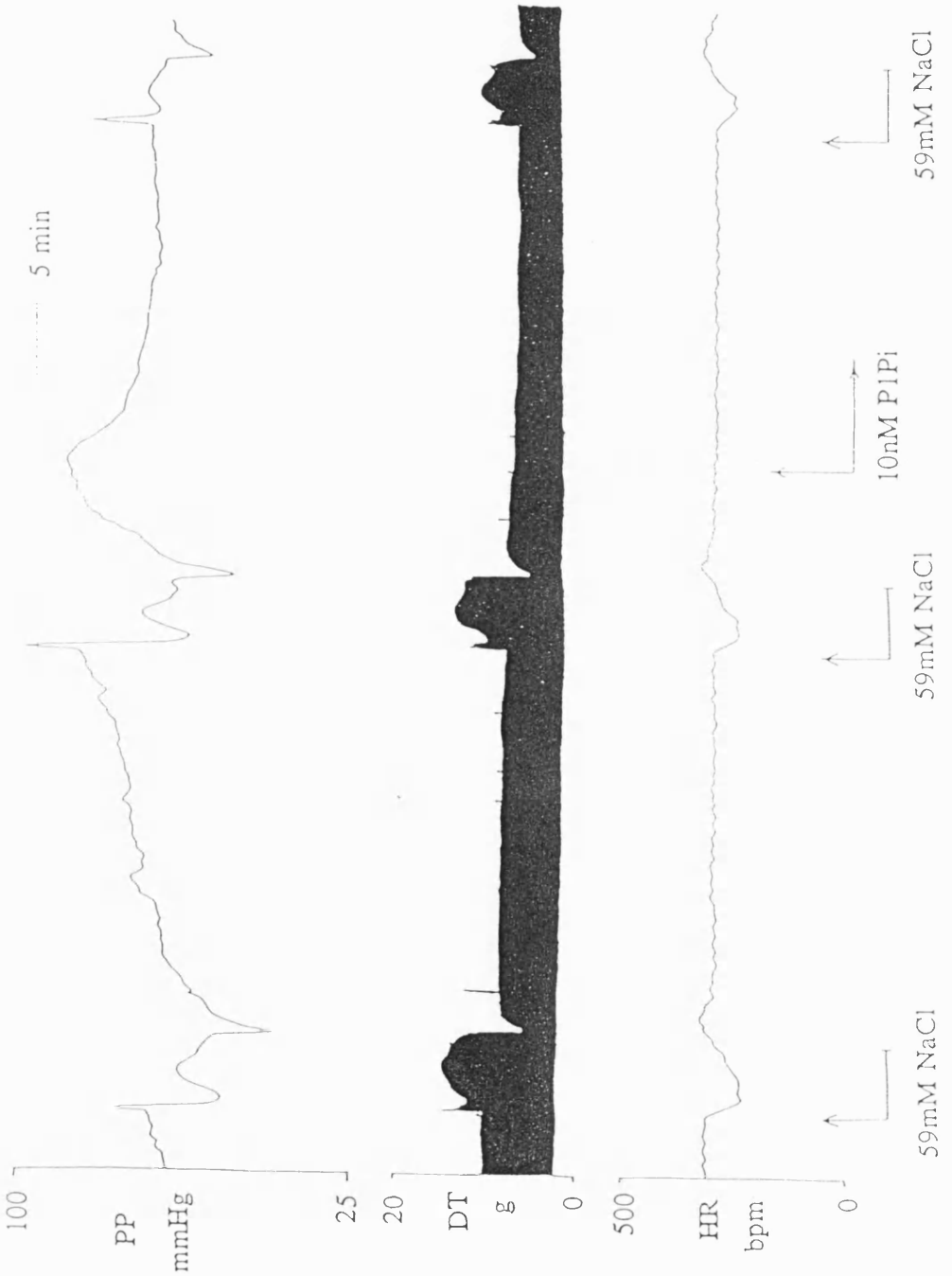


**1.8.2 Effect of P1Pi on the actions of a low sodium (59mM NaCl) perfusate on developed tension, heart rate and perfusion pressure.**

A concentration of 59mM NaCl was used, as this was found to produce a submaximal increase in developed tension in the previous experiment. Perfusing hearts with the low sodium solution for 5 minutes at 20 minute intervals induced a positive inotropic response, bradycardia and a complex effect on perfusion pressure consisting of a small coronary constriction followed by a coronary dilator component (Figure 36). Third low sodium perfusion responses are quoted, because in control preparations these responses were found to be not significantly different to second responses. In control hearts the third low sodium perfusion period produced a developed tension of $163\pm12.7\%$ of its resting value (n=6) while in the presence of P1Pi (10nM) developed tension was $164\pm7.2\%$ of the resting value (n=8) ie. not significantly different from the control response. The decrease in heart rate induced by third low sodium perfusion periods in control and P1Pi (10nM) treated hearts were $47\pm9$ and $59\pm7$ bpm respectively (n=6-8), these decreases were not significantly different. Basal heart rates in these control and P1Pi treated hearts were $286\pm10$ and $294\pm13$ bpm respectively (n=6-8). Coronary perfusion pressure changes induced by the third low sodium perfusion period in control hearts consisted of an initial coronary constriction which increased $14\pm0.4$mmHg above the basal perfusion pressure of $76\pm3.8$mmHg (n=6). This was followed by a fall in pressure which reached a value of $11\pm2.2$mmHg below the basal perfusion pressure, ie. from its peak value, perfusion pressure fell by an average of 25mmHg. In the presence of P1Pi (10nM) basal perfusion pressure was $65\pm4.6$mmHg (n=8) and the equivalent increase in perfusion pressure due to low sodium perfusion was $13\pm2.7$mmHg (n=8), an effect which was not significantly different from the control response. However the coronary dilator component of the response to low sodium was significantly ($p<0.05$) attenuated in the P1Pi treated hearts. In the presence of P1Pi the perfusion pressure fell $0.1\pm0.1$mmHg (n=8) below the basal value compared with $11\pm2.2$mmHg (n=6) in the control experiments.

Figure 36

Trace showing the effect of P1Pi on the developed tension (DT), heart rate (HR) and perfusion pressure (PP) responses to low sodium (59mM) perfusion in the isolated perfused rat heart. P1Pi was perfused prior to, and during, the third low sodium perfusion period.



## 1.9. The effect of P1Pi or palmitoyl carnitine on the effects of 3x 20 minutes zero flow global ischaemia / reperfusion in the isolated heart.

The ability of P1Pi to inhibit the actions of mediators such as catecholamines and prostaglandins which are known to be released following ischaemia, and which may contribute to ischaemically-induced damage (Ceremuzynski et. al., 1969; Berger et al., 1976), raises the possibility that P1Pi could be protective under conditions of ischaemia / reperfusion. In addition, both potassium channel openers (Grover et al., 1989; Gross & Auchampach, 1992) and calcium antagonists (Watts et al., 1986) have been shown to produce protective effects in global ischaemia / reperfusion models, and P1Pi has shown characteristics in common with both these classes of compound. Therefore these experiments were performed in order to investigate whether P1Pi could modify the ischaemic contracture or depression of developed tension produced by zero flow global ischaemia in the isolated rat heart. The actions of P1Pi were compared with the actions of the parent compound palmitoyl carnitine.

### 1.9.1 The effect of 3x 20 minutes global zero flow ischaemia / reperfusion on developed tension, heart rate and perfusion pressure in the isolated rat heart.

Figure 37a is a representative trace showing the effect of 20 minutes zero flow global ischaemia / reperfusion, repeated three times on perfusion pressure, heart rate and developed tension in the isolated rat heart. During the first 20 minute period of ischaemia, the developed tension dropped off slowly and a small degree of ischaemic contracture developed. Heart rate initially increased slightly, possibly due to impairment of the triggering of the rate meter, but heart rate was lost as the ischaemic contracture developed. On the first period of reperfusion, an immediate increase in coronary perfusion pressure was obtained, which was followed by a rapid fall in perfusion pressure, and subsequently a gradual coronary constriction developed starting at approximately 5 minutes after reperfusion. Developed tension was re-

established on reperfusion but the force of contraction was reduced compared to that before ischaemia. There was no difference in heart rate before or after 20 minutes ischaemia. In subsequent ischaemic periods, the ischaemic contracture increased and in subsequent periods of reperfusion, there was a further depression of developed tension and coronary constriction developed more rapidly.

Figures 38 and 39 show in graphical form the effect of 3x 20 minutes ischaemia / reperfusion on contracture developed during each ischaemic period and the developed tension during each period of reperfusion respectively.

### 1.9.2 The effect of P1Pi on ischaemic contracture, and developed tension and perfusion pressure during reperfusion, produced by 3x 20 minutes ischaemia / reperfusion in the isolated rat heart.

Figure 37b is a representative trace of the effect of 100nM P1Pi on the effect of 3x 20 minutes ischaemia / reperfusion in the isolated rat heart. At the lower concentration of 10nM, P1Pi had no effect on ischaemic contracture or depression of developed tension during reperfusion (Figure 38 & 39). However, in the presence of 100nM P1Pi, the contracture developed was slightly, but not significantly, suppressed during the first 20 minutes ischaemia. There was no significant effect of P1Pi on subsequent periods of ischaemia (Figure 38). At neither concentration was there any effect of P1Pi on depression of developed tension following reperfusion (Figure 39).

At a concentration of 100nM, however, P1Pi significantly ($p < 0.05$) inhibited the slowly developing coronary constriction produced during each period of reperfusion (Figures. 37 & 40).

### 1.9.3 Effect of palmitoyl carnitine on ischaemic contracture, and developed tension and perfusion pressure during reperfusion, produced by 3x 20 minutes ischaemia / reperfusion in the isolated rat heart.

Palmitoyl carnitine at a concentration of 100nM had no significant effect on ischaemic contracture, or developed tension during reperfusion in these experiments (Figure 38 & 39). The slowly developing coronary constriction, commencing after approximately 5 minutes reperfusion, was also not significantly affected by 100nM palmitoyl carnitine (data not shown).

Figure 37a

Trace showing the effect of 3x 20 minutes zero flow global ischaemia / reperfusion on developed tension, heart rate and perfusion pressure in the isolated perused rat heart.

Figure 37b

Trace showing the effect of 3x 20 minutes zero flow global ischaemia / reperfusion on developed tension, heart rate and perfusion pressure in the isolated perfused rat heart in the presence of 100nM P1Pi.

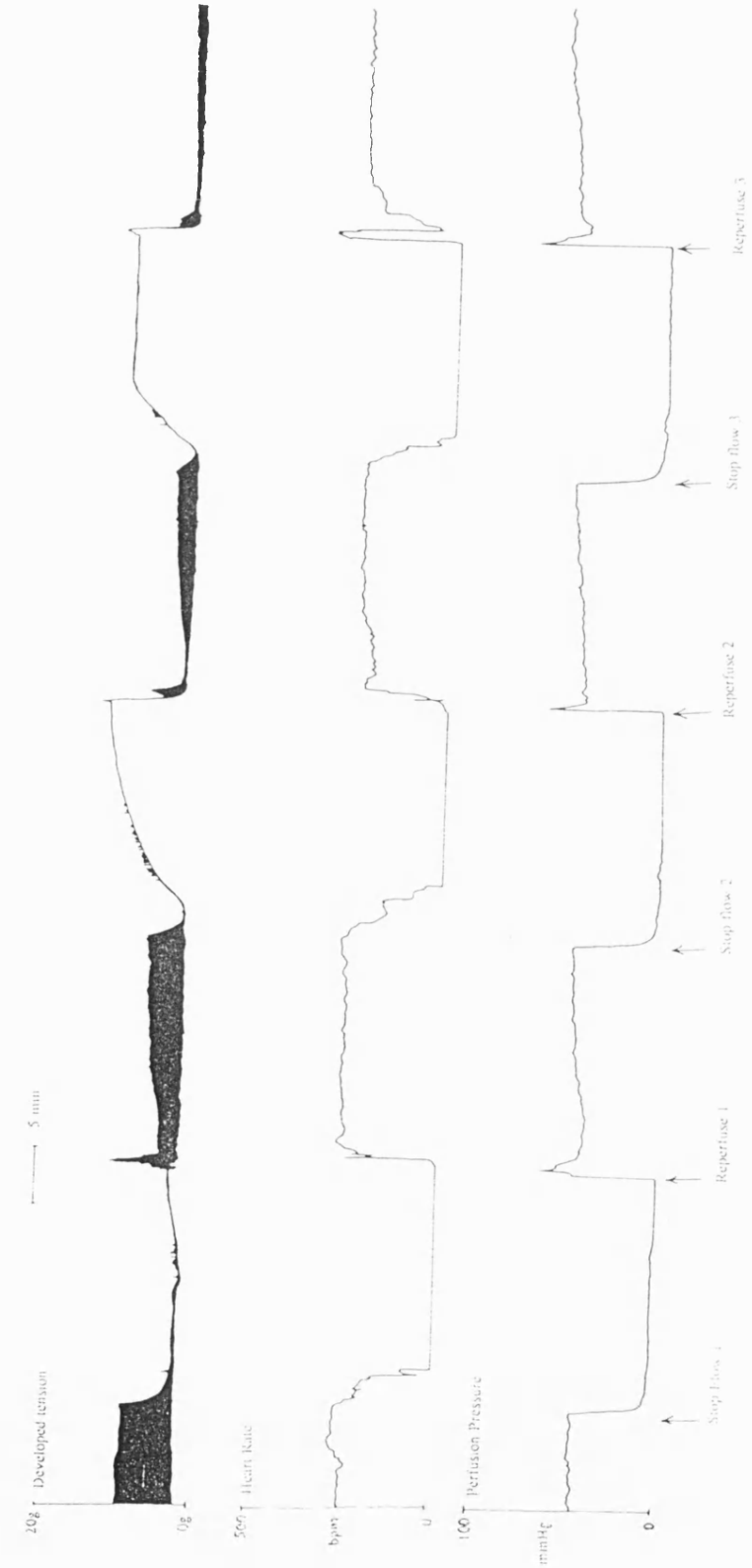


Figure 38

The effect of P1Pi and palmitoyl carnitine (PC) on the three periods of ischaemic contracture developed during 3x 20 minutes zero flow global ischaemia in the isolated, perfused rat heart. Data expressed as basal (diastolic) tension (g).

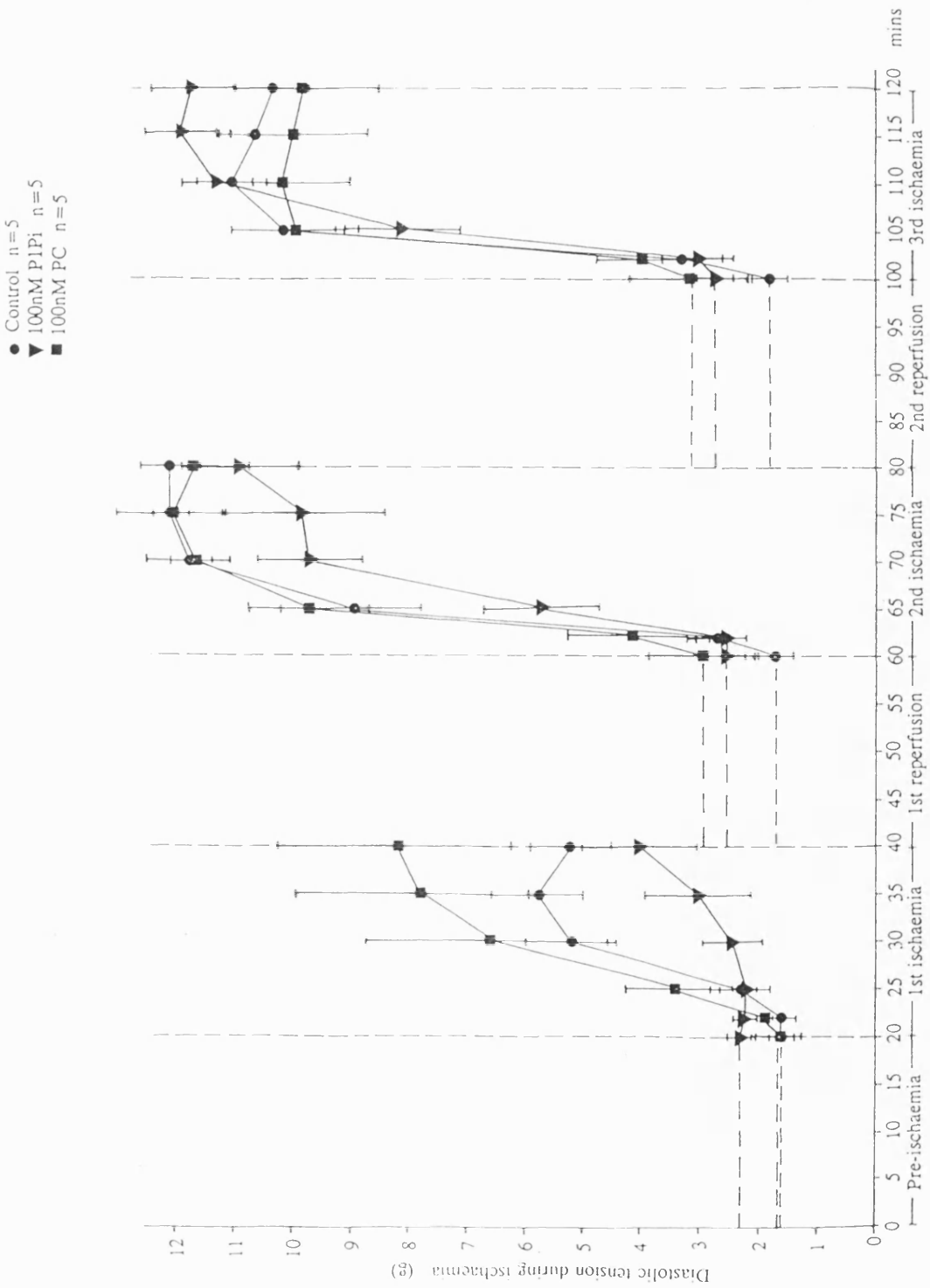


Figure 39

The effect of P1Pi and palmitoyl carnitine (PC) on the 3x 20 minute periods of reperfusion, following 20 minutes zero flow global ischaemia in the isolated, perfused rat heart. Data expressed as developed tension (g).

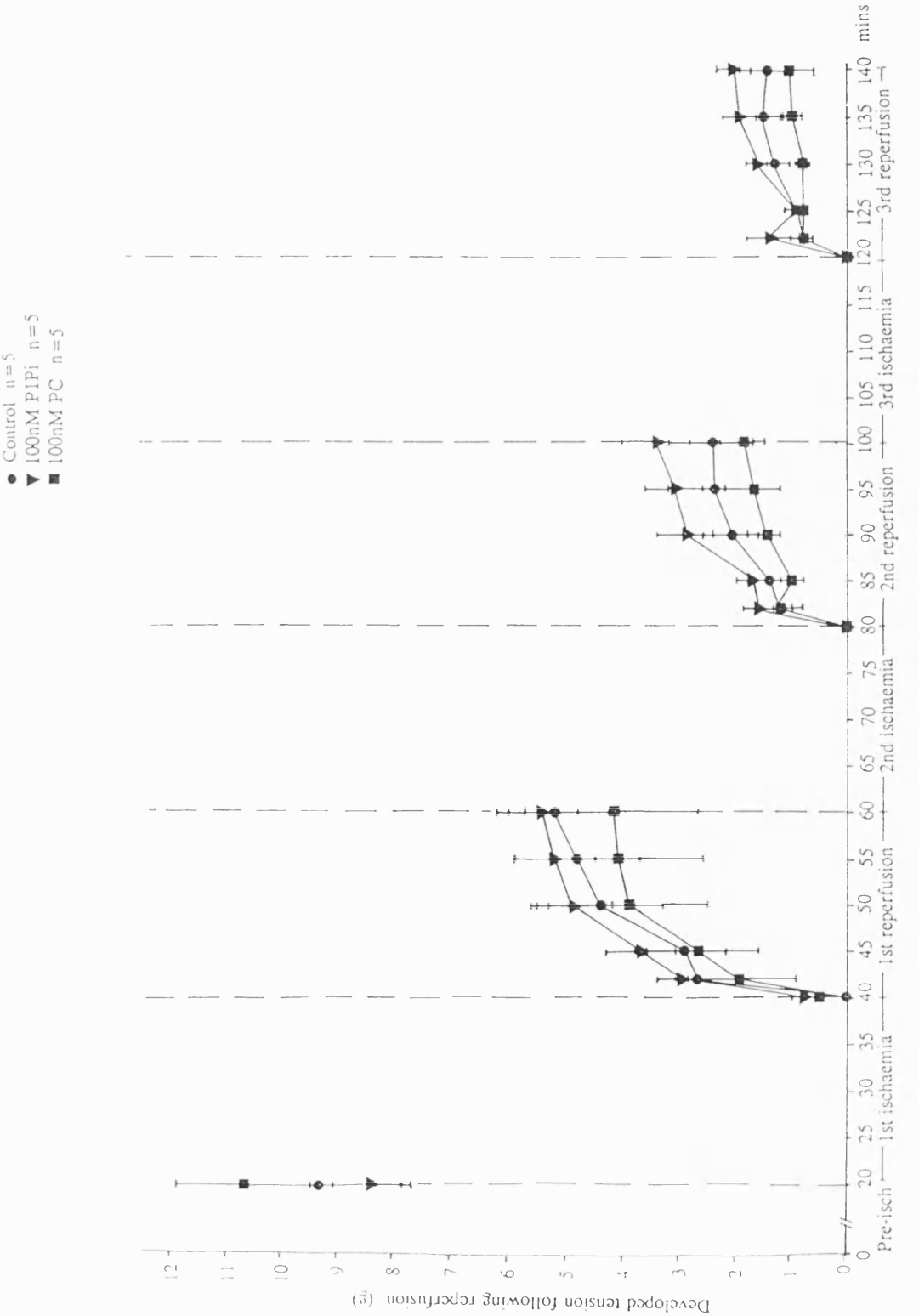
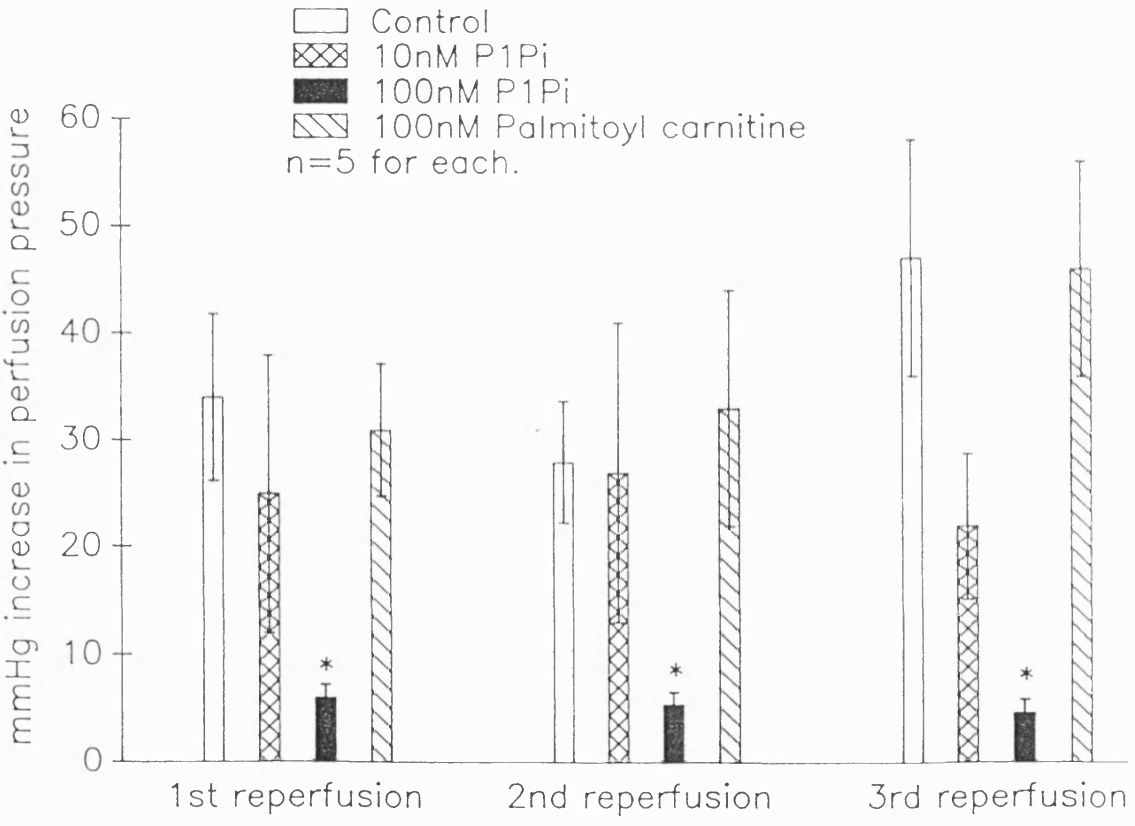


Figure 40

The effect P1Pi and palmitoyl carnitine on the increase in perfusion pressure during reperfusion following 3x 20 minutes ischaemia in the isolated perfused rat heart. Data expressed as mmHg increase in perfusion pressure gained between 5 and 15 minutes reperfusion.

Basal perfusion pressure: Control 80±5.8mmHg; 10nM P1Pi 77±9.2mmHg; 100nM P1Pi 50±3.4mmHg; 95±11mmHg, n=5 for each, *$p<0.05$ compared with control increases, 1 way ANOVA.



## 2. Isolated, stimulated left atrium of the rat.

### 2.1 Effect of P1Pi on the increase in developed tension in response to noradrenaline in the isolated rat left atrium, stimulated at 0.5Hz.

The use of the isolated perfused hearts to investigate the mechanism of action of P1Pi poses the problem of interaction between vascular and myocardial contractile effects. In order to isolate the positive inotropic effects of noradrenaline from effects on the coronary vasculature, the effect of P1Pi on the positive inotropic action of noradrenaline in the isolated left atrium of the rat was investigated.

Noradrenaline (100nM - 30$\mu$M) added cumulatively to the organ bath produced a graded increase in the height of the twitch response in response to electrical stimulation at 0.5Hz. On washing out and repeating the concentration effect curve at 20 minute intervals, there was no diminution of the responses (Figure 41).

The addition of P1Pi at concentrations of 100nM and 1$\mu$M had no significant effect on the basal twitch height; in time-matched control curves the resting developed tension was 53$\pm$14mg n=4, in the presence of 100nM P1Pi this was 83$\pm$17mg n=4 and in the presence of 1$\mu$M P1Pi the basal developed tension was 60$\pm$7.1mg n=5. It can be seen from Figures 41 & 42 that P1Pi at concentrations of 100nM and 1$\mu$M had no significant effect on the noradrenaline-induced increase in developed tension in the isolated left atrium (Figures 41 & 42).

Figure 41

Trace showing the effect of noradrenaline in the isolated left atrium of the rat, stimulated at 0.5Hz, 1ms, 50% above the threshold voltage, both in the absence and presence of P1Pi.

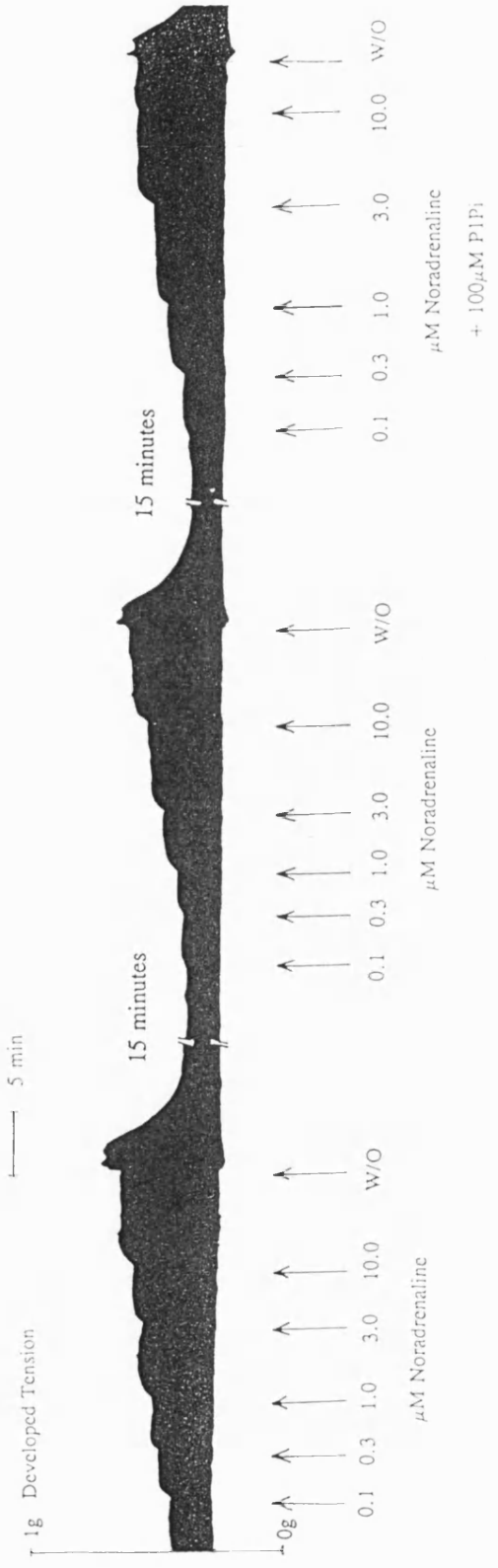
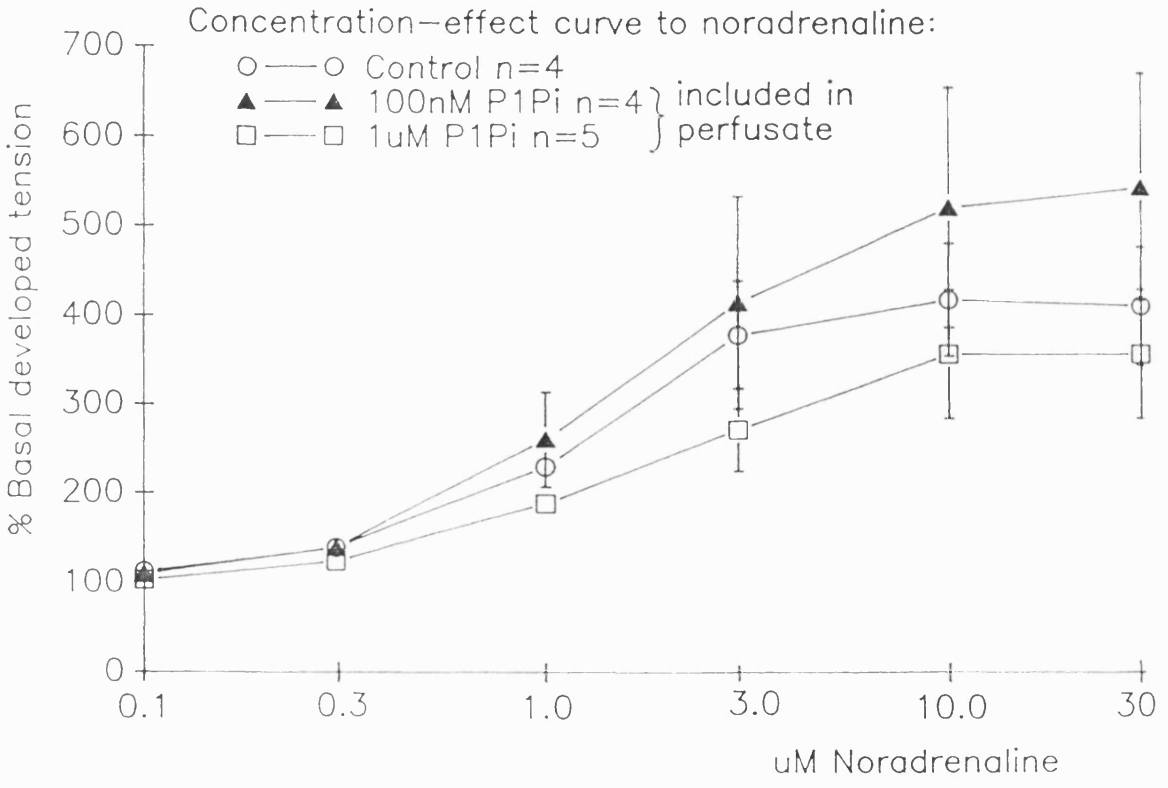


Figure 42

The effect of P1Pi on the increase in developed tension produced in response to noradrenaline in the isolated, stimulated left atrium of the rat. Data expressed as % of the basal developed tension, third concentration effect curves shown, in the absence or presence of P1Pi.

Basal developed tension: Time matched control 53±14mg n=4, 100nM P1Pi 83±17mg n=4, 1$\mu$M P1Pi 60±7.1mg n=5.



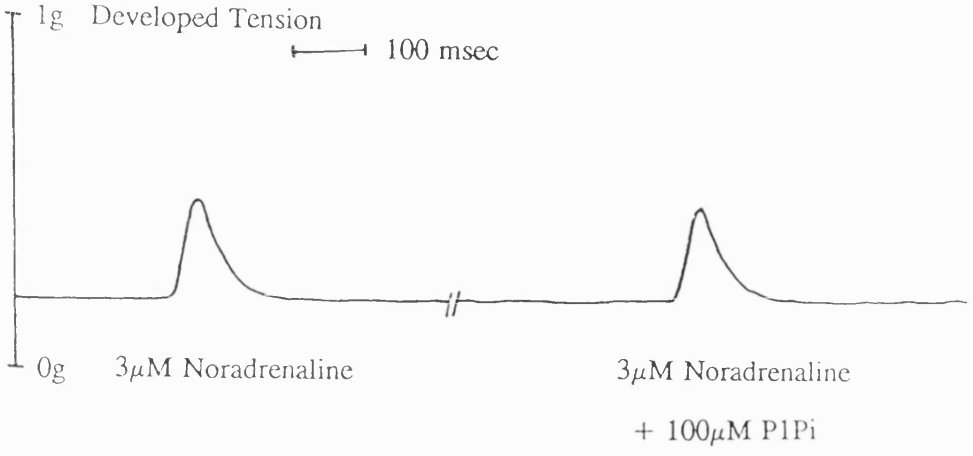
**2.2 Effect of P1Pi on the length of the twitch response in the isolated stimulated left atrium in the presence of noradrenaline.**

In order to investigate whether P1Pi could affect the twitch shortening produced in response to noradrenaline, the effect of P1Pi on twitch length in the presence of a submaximal concentration (3$\mu$M) of noradrenaline in the isolated stimulated left atrium was investigated.

Figure 43 shows the effect of P1Pi (100nM) on the twitch response in the presence of 3$\mu$M noradrenaline in the isolated left atrium, stimulated at 0.5Hz. It can be seen that this concentration of P1Pi is not able to affect either the twitch height or the twitch length. In time-matched control preparations, the length of the twitch response was 285$\pm$21.8msec after the second and 272$\pm$21.3msec, n=4 after the third application of noradrenaline. In test preparations, the second application of noradrenaline (in the absence of P1Pi) produced a twitch length of 191$\pm$6.6msec and in the presence of 100nM P1Pi (third application) a twitch length of 178$\pm$3.2msec, n=4.

Figure 43

Trace of a single twitch (100mm/sec) in the isolated left atrium stimulated at 0.5Hz in the presence of noradrenaline, and effect of P1Pi on this response.



### 3. The isolated rat ventricular myocyte preparation.

In view of the ability of P1Pi to suppress agonist-induced positive inotropic responses in whole hearts, and the lack of effect of P1Pi on positive inotropic responses in isolated atria, these experiments were performed in order to investigate whether P1Pi could affect calcium fluxes in isolated myocytes. This was achieved by measuring intracellular free calcium using fura-2 fluorescence measurements.

During the enzymic digestion of the rat heart, perfusion pressure was initially $53 \pm 2.4$mmHg prior to the addition of collagenase ($n=30$). This increased to a maximum of $110 \pm 4.4$mmHg on addition of 20mg collagenase to the recirculating solution. Following 20 minutes enzymic digestion, the perfusion pressure fell back to a mean of $37 \pm 3.0$mmHg.

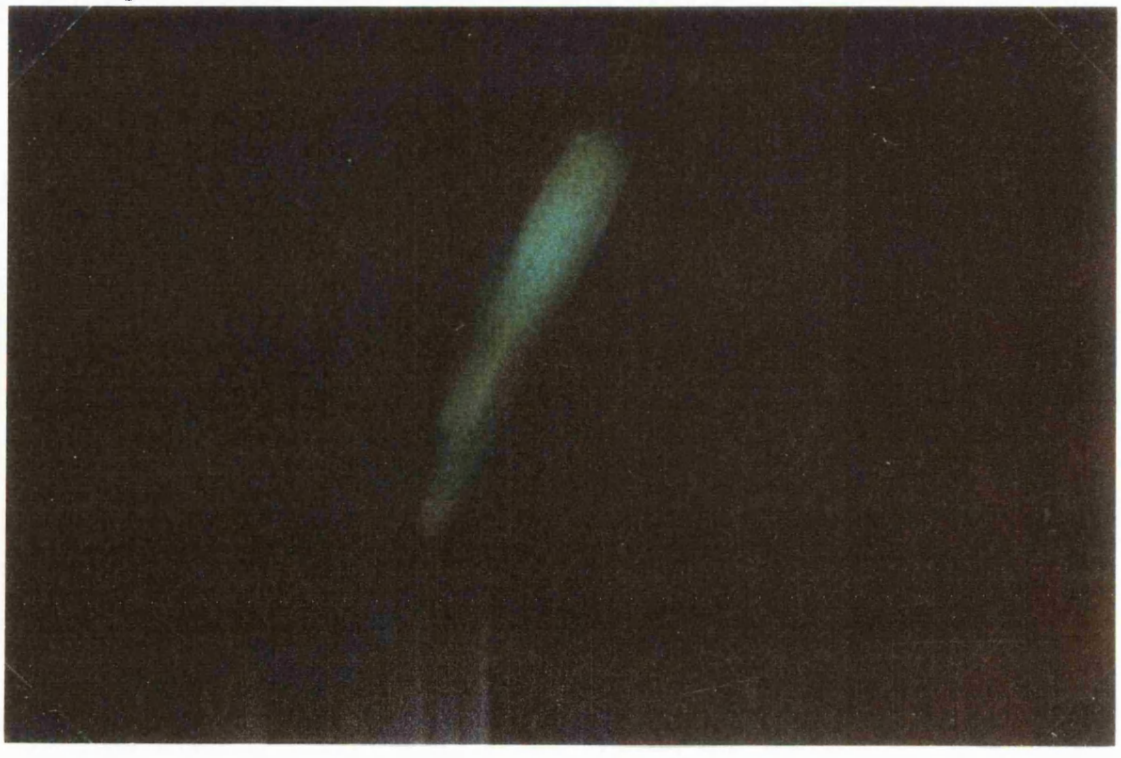
Figure 44 shows a typical population of rat ventricular myocytes, at x400 magnification, following the isolation procedure described in the methods section. Typically, 60-80% viability was obtained. Cells which were rod-shaped, quiescent and showing clear striation were selected for fluorimetric measurements. Figure 45 shows the fluorescence emitted from one such cell loaded with fura-2 and excited at wavelengths of 340/380nm.

Figure 44

Print of rat myocytes, x400 magnification, isolated according to the technique described.

Figure 45

Print of a single myocyte, x400 magnification, loaded with fura-2 and excited at wavelengths 340/380 nm.



**3.1 Effect of electrical stimulation on the 340/380 fura-2 signal ratio in a single, isolated myocyte.**

Shown in Figure 46a is a representative trace of the effect of electrical stimulation at 1Hz, 1ms duration and at 50% above the threshold voltage, whilst superfusing a single isolated cardiac myocyte at 2ml/min with 1mM $Ca^{2+}$ at room temperature, on the 1A and 2A signals. These represent the emission at 510nm from excitation wavelengths of 340nm and 380nm respectively. With increasing free calcium within the cell, the emission at 510nm from the excitation wavelength of 340nm (1A) increases, and from the excitation wavelength of 380nm (2A) the emission at 510nm decreases. Therefore, the 340/380 signal ratio is directly proportional to intracellular free calcium, and this ratio is represented in Figure 46b, obtained from the 1A and 2A signals shown in Figure 46a. It can be seen that each electrical stimulus, at 1 second intervals, produces a several fold increase in the 340/380 fura-2 signal ratio which rapidly falls back to the basal ratio prior to the next electrical stimulus. These increases indicate calcium transients occurring within the cell, due to membrane depolarisation in response to the electrical stimulus. The mean basal 340/380 ratio was $0.76 \pm 0.03$, increasing to a maximum of $2.04 \pm 0.14$ following each electrical stimulus ($n = 14$).

Figure 46a

The 510nm emission signal from a single myocyte alternately excited at 340nm (1A) and 380nm (2A) wavelengths (60Hz).

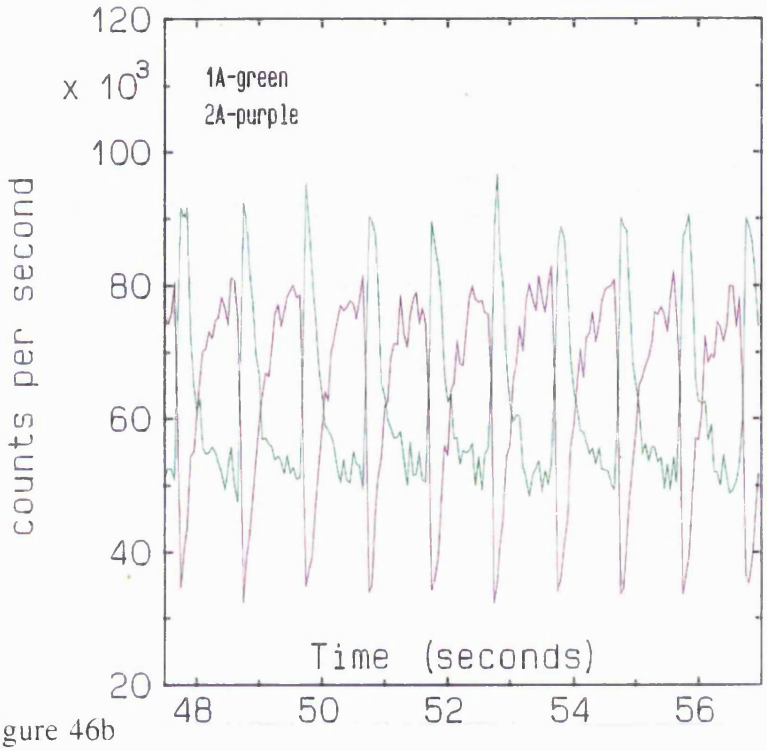
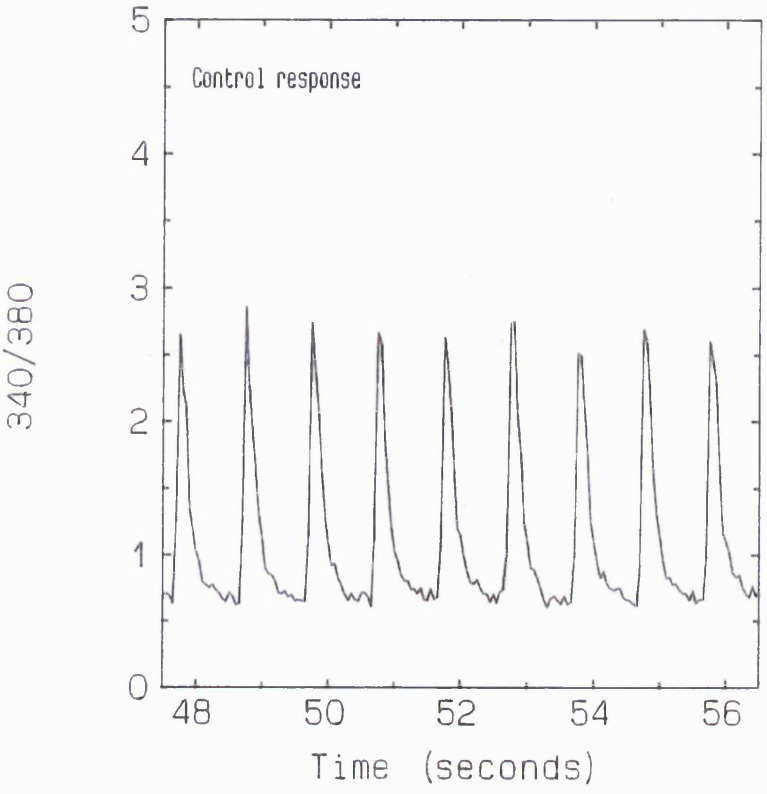



Figure 46b

The fura-2 340/380 ratio signal, proportional to intracellular free calcium concentration.



### 3.1.1.1 Effect of noradrenaline on the 340/380 signal ratio increase in response to electrical stimulation at 1Hz in a single myocyte.

Figure 47a is a representative trace showing the effect of 300nM noradrenaline in the superfusate on the 340/380 fura-2 signal ratio in a single myocyte stimulated at 1Hz. Following the addition of noradrenaline to the superfusate at 60 seconds, there was a slowly developing response which reached a maximum after approximately 90 seconds. It can be seen from Figure 47a that in the presence of 300nM noradrenaline, 150-155 seconds on the time scale, the 340/380 signal ratio increase, representing calcium transients, is considerably increased. The 340/380 ratio increase was measured from the resting value to the maximum value in response to each electrical stimulus; the average of 5 peaks being measured from 50-55s for control levels, and 150-155s for transients in the presence of 300nM noradrenaline for each myocyte. In the presence of 300nM noradrenaline, the ratio increases in response to electrical stimulation were significantly increased; the ratio increase being $174 \pm 10.9\%$ of the ratio increase in the absence of noradrenaline ($n = 14$ myocytes). The resting 340/380 signal ratio was $0.76 \pm 0.03$ before the addition of 300nM noradrenaline and $0.84 \pm 0.04$ in the presence of 300nM noradrenaline in the superfusate, therefore there was no significant change in the resting ratio and thus resting calcium concentration. However, the maximum 340/380 signal ratio, and thus elevated calcium concentration in response to each stimulus, was significantly increased from $2.04 \pm 0.14$ to $3.11 \pm 0.42$, $n = 14$. It can also be seen from Figure 47b that the duration of the calcium transients is shortened in the presence of 300nM noradrenaline. A lower concentration of noradrenaline in the perfusate ie. 30 or 100nM was found to have no significant effect on the 340/380 signal ratio increase in response to stimulation at 1Hz. Concentrations above 300nM noradrenaline did not produce any further increase in the 340/380 signal ratio increase in response to electrical stimulation (data not shown). Therefore, no graded response to noradrenaline could be obtained and this was compounded by the wide variation in responses between preparations.

Figure 47a

Traces showing the fura-2 340 /380 signal ratio increases, representing calcium transients, in response to electrical stimulation at 1Hz, in the absence (50-55seconds) and presence (150-155seconds) of 300nM noradrenaline in a single myocyte, superfused at 2ml/min.

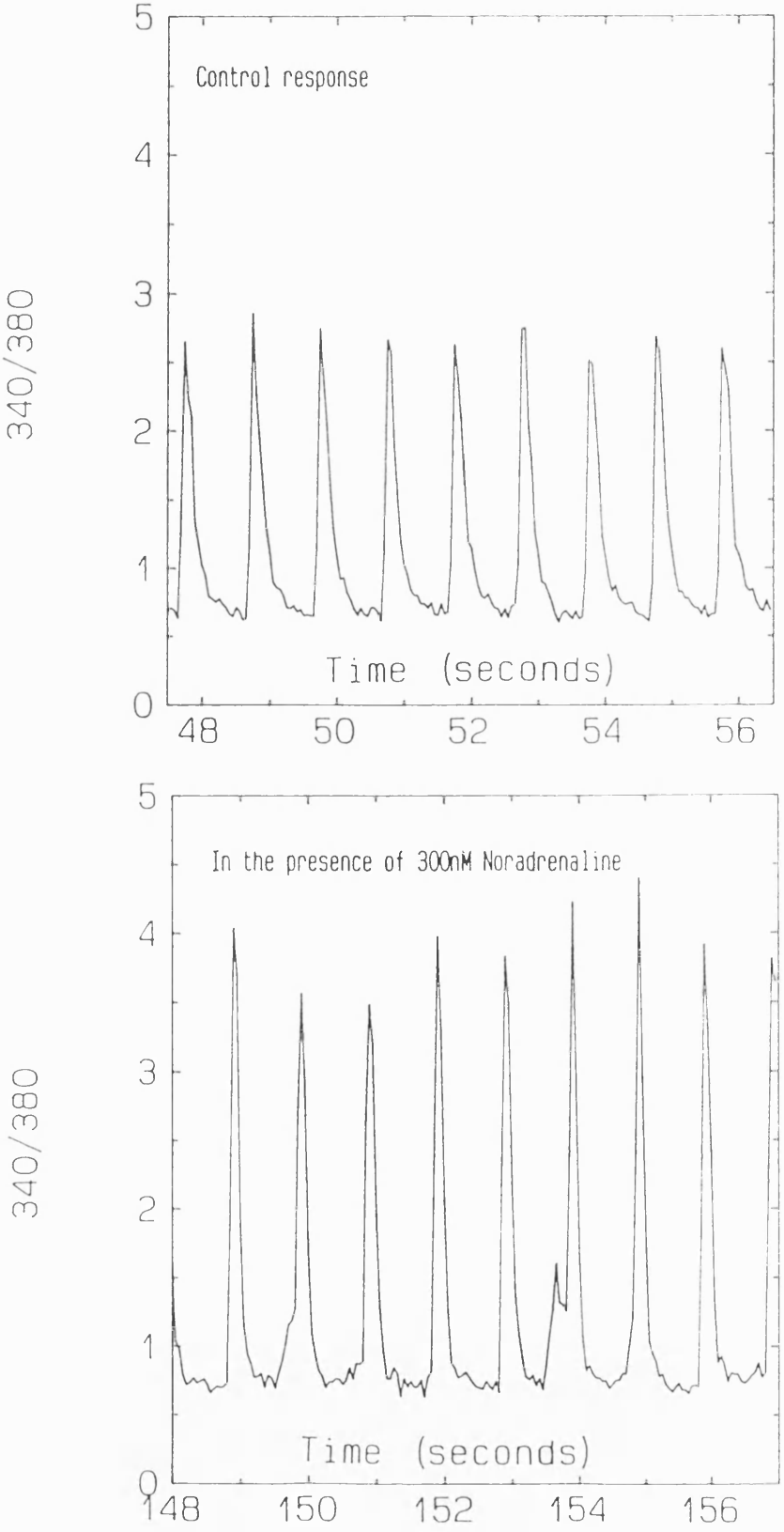
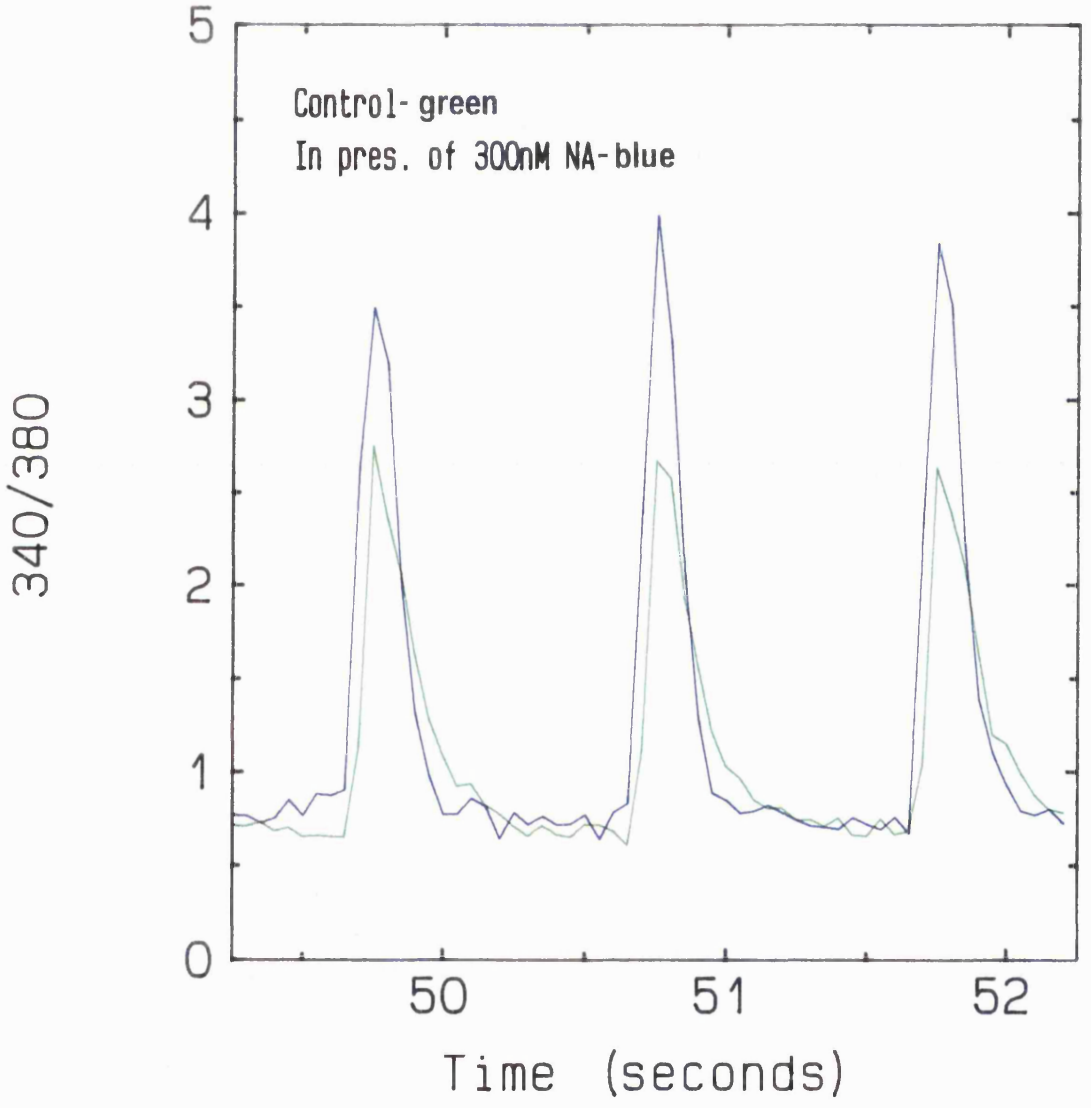


Figure 47b

Trace showing 3 calcium transients from Figure 47a, transients in the presence of 300nM noradrenaline (NA) superimposed on control transients (time scale for transients in presence of noradrenaline = time shown + 100 seconds).



### 3.1.2 Effect of P1Pi on basal 340/380 signal ratio increases.

P1Pi had no significant effect on basal 340/380 signal ratio increases in response to electrical stimulation at 1Hz. The ratio increase in the presence of P1Pi was found to be 96±7.0% of that in the absence of P1Pi (n=5). However in the presence of P1Pi, there was a consistent increase in the 'noise' on the trace, ie. the calcium transients were found to be less smooth than in the absence of P1Pi (see fig. 48). The duration of the calcium transients in the presence of P1Pi also appeared shorter compared to control responses, but this was difficult to measure because of the increased 'noise'.

### 3.1.3 Effect of P1Pi and atenolol on the 340/380 signal ratio increase in response to noradrenaline.

Figure 48 is a representative trace of the effect of noradrenaline on the 340/380 signal ratio increases in response to electrical stimulation at 1Hz, in the presence of 100nM P1Pi. The presence of 100nM P1Pi in the superfusate produced a significant inhibition of the 300nM noradrenaline-induced % increase in the calcium transients, measured by 340/380 signal ratio changes (Figure 49). However, the noradrenaline-induced shortening of the calcium transients did not appear to be affected by P1Pi (Figure 48b). As with P1Pi, atenolol (1$\mu$M) also significantly inhibited the noradrenaline (300nM) induced increases in the calcium transients (Figure 49).

Figure 48a

Trace showing the fura-2 340/380 signal ratio, representing $Ca^{2+}$ transients in a single myocyte superfused at 2ml/min in response to electrical stimulation at 1Hz, in the absence (50-55s) and presence (150-155s) of 300nM noradrenaline, 100nM P1Pi perfused throughout the experiment.

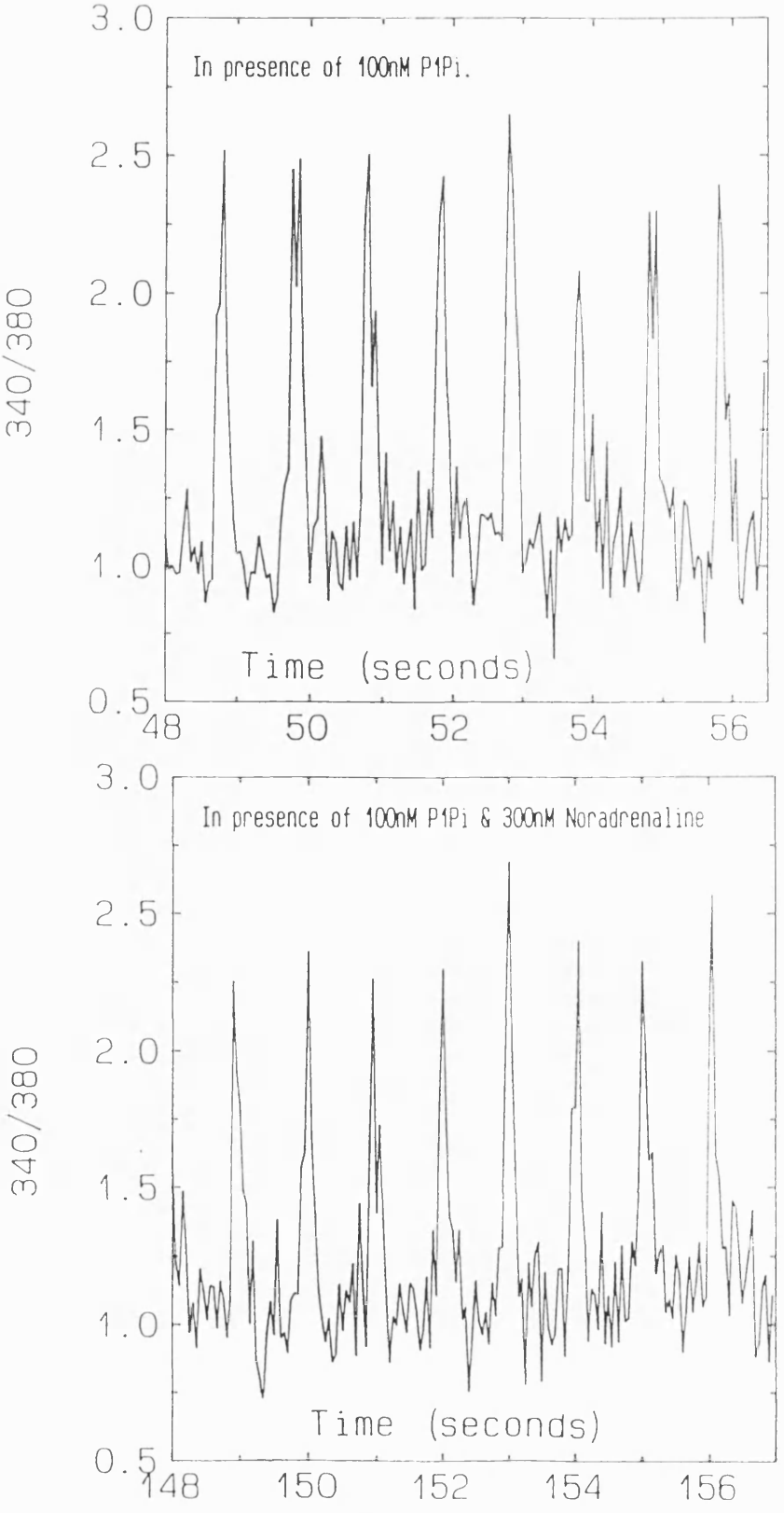


Figure 48b

Trace showing 3 calcium transients from Figure 48a, transients in the presence of P1Pi and noradrenaline (NA) superimposed on transients in the presence of 100nM P1Pi only (time scale for transients in presence of both noradrenaline and P1Pi = time shown + 100 seconds).

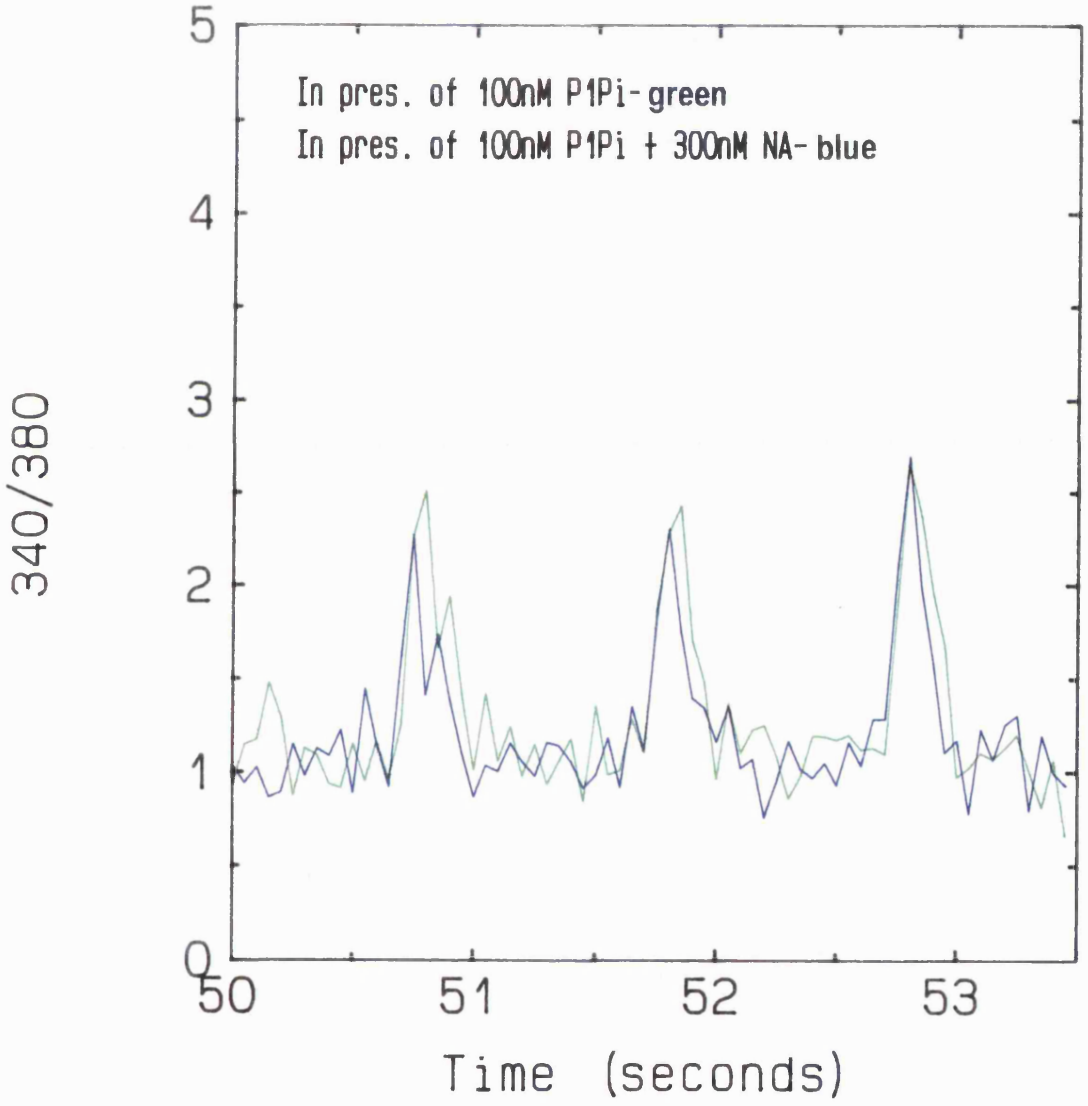
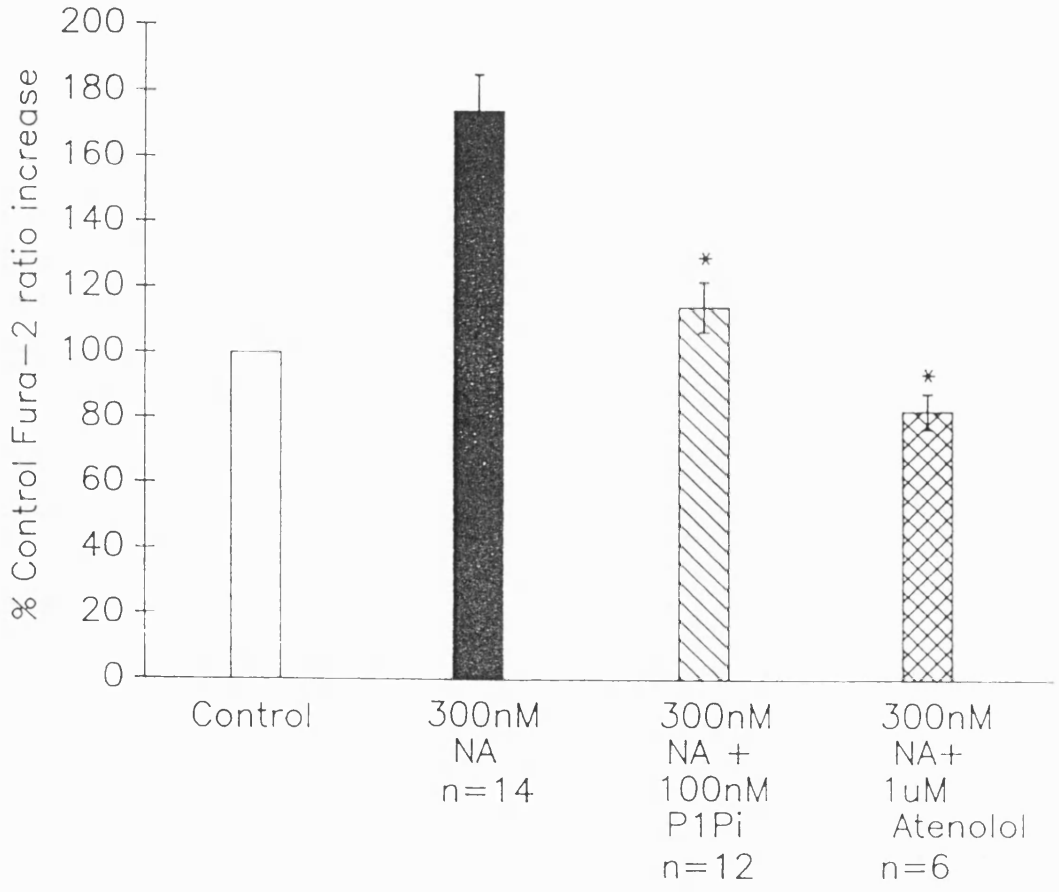


Figure 49

The effect of P1Pi and atenolol on the increase in the 340/380 signal ratio in response to noradrenaline (NA). Data expressed as a % basal 340/380 signal ratio increase. For each myocyte the mean of 5 peaks from 50-55seconds in the absence of noradrenaline (basal) and from 150-155seconds in the presence of noradrenaline were used to calculate the % increases. *$p < 0.05$, compared to increases in the absence of P1Pi or atenolol, 1 way ANOVA.



### 3.1.1.2 Effect of verapamil on the 340/380 signal ratio increase in response to electrical stimulation at 1Hz.

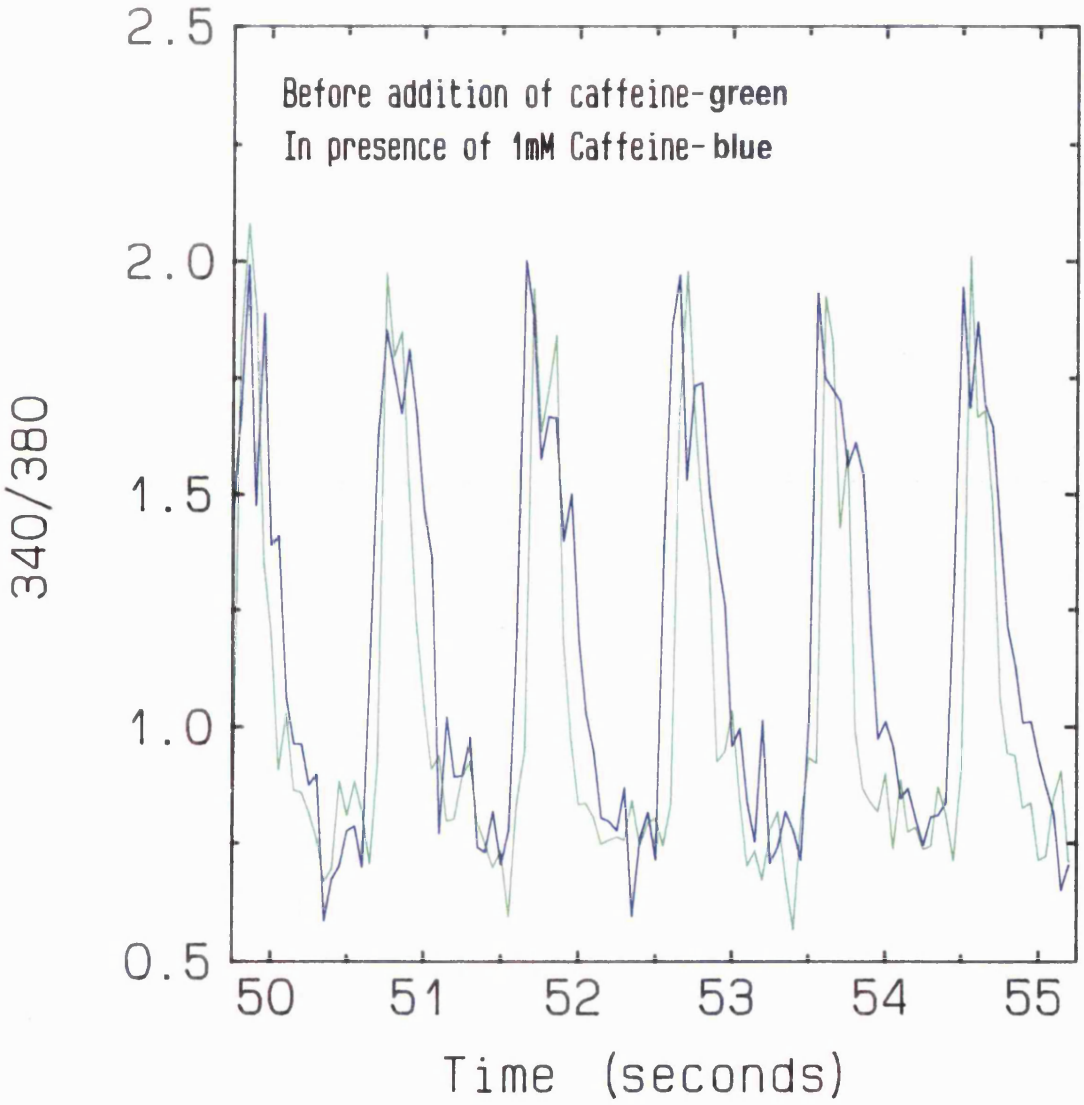
A concentration of 10$\mu$M verapamil was required to completely inhibit the $Ca^{2+}$ transients (340/380 signal ratio) +
produced in response to 1Hz electrical stimulation (data not shown). Lower concentrations of verapamil had no significant effect on the calcium transients produced in response to electrical stimulation.

### 3.1.1.3 Effect of caffeine on the 340/380 signal ratio increase in response to electrical stimulation at 1Hz.

Figure 50 is a trace showing the effect of perfusing 1mM caffeine on the 340/380 signal ratio increase, representing calcium transients, in response to electrical stimulation. This trace is representative of 8 similar experiments. It can be seen that while caffeine has no effect on the amplitude of the oscillations, the duration of the transients is increased. A concentration of 100nM P1Pi appeared to have no effect on the increase in duration of the calcium transients (n=3, data not shown).

Figure 50

Trace showing the effect of caffeine on the fura-2 340/380 signal ratio increase in response to electrical stimulation at 1Hz, in a single myocyte, superfused at 2ml/min. (Time scale in presence of caffeine = time shown + 100 seconds).



## Discussion

These studies have primarily investigated the actions of novel derivatives of palmitoyl carnitine in the coronary vasculature and myocardium.

### 1. Structure-activity relationship studies, and the effect of verapamil on coronary constrictor and dilator responses to compound G.

The initial studies described involved the investigation into structure-activity relationships of various synthetic derivatives of palmitoyl carnitine in the isolated, perfused rat heart in order to elucidate the groups of the molecule required for the generation of a coronary dilator response.

### 1.1 Importance of the C terminus ester group on coronary dilator activity.

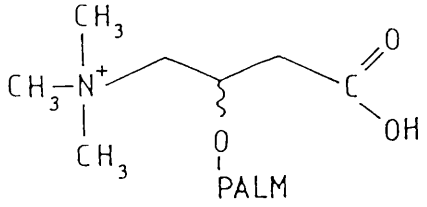
The parent molecule, palmitoyl carnitine (See Table 1 below for structures) has previously been shown to be a potent coronary constrictor in the isolated perused rat heart (Criddle et al., 1990). The conversion of this zwitterionic amphiphile to a cationic ester derivative, by the action of an alcohol, has been shown to convert the molecule from a coronary constrictor to a coronary dilator (Criddle et al., 1990). The present study shows that the presence of an ester grouping is not necessary for the coronary dilator response. Compound A differs from the parent compound palmitoyl carnitine in the presence of an ethyl group at the C terminus, in place of a carboxylic acid group (see Table 1, Results). Like the isopropyl ester derivative of palmitoyl carnitine (P1Pi), Compound A was found to be an active coronary dilator in the coronary circulation (Figures 8, 9 & 10). Thus it appears that the absence of a negatively charged carboxylic acid group, producing a molecule with an overall

positive charge is the necessary requirement in the conversion of the molecule from a coronary constrictor to a coronary dilator. However, Compound A produced a more pronounced transient coronary constriction preceding the dilator response than P1Pi, which may account for to the significantly smaller coronary dilator responses to lower doses of Compound A than equivalent doses of P1Pi.

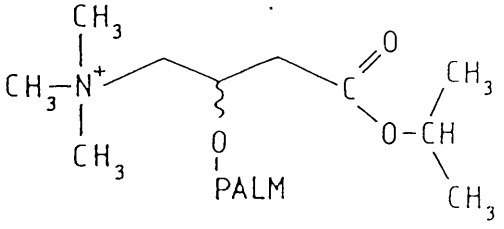
**Table 1**

Structures of the palmitoyl carnitine derivatives used in this study.

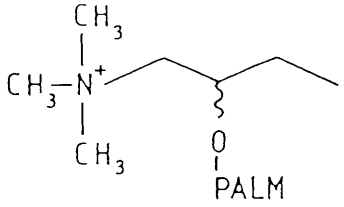
Palmitoyl carnitine



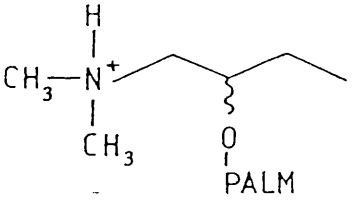

Palmitoyl carnitine Isopropyl ester (P1Pi)



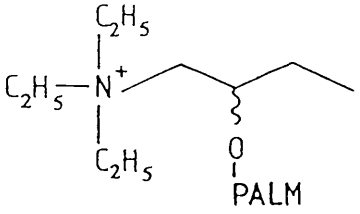

Compound A



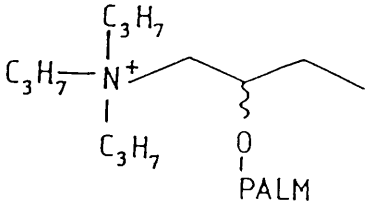

Compound B



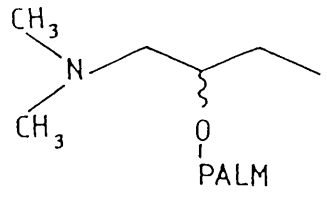

Compound C




Compound D




Compound E




Compound F

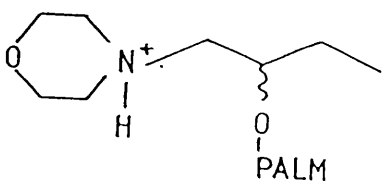
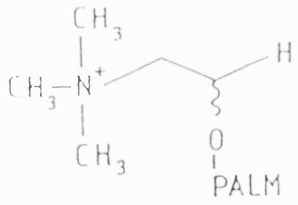
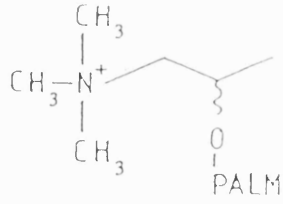


Table 1 cont.

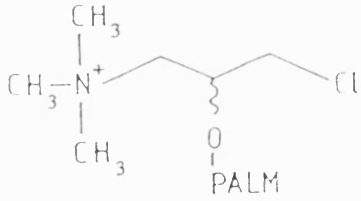
Compound G



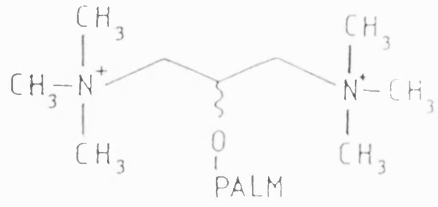

Compound H



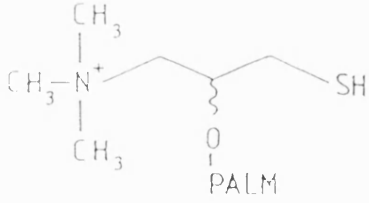

Compound I



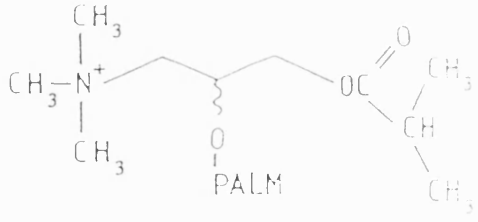

Compound J



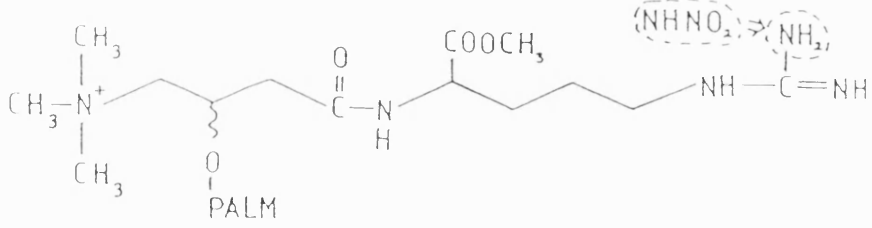

Compound K



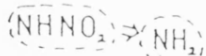

Compound L




Compound M




Compound N



Further structure-activity relationship studies were carried out in order to establish the groups attached at the N and C terminus of the carnitine moiety producing the optimum coronary dilator potency.

**1.2 Effect of N terminus substitution on coronary dilator potency.**

With regard to N terminus groups, the size of the groupings attached to the nitrogen considerably affect the charge on the nitrogen. As Compound E, containing only 2 (methyl) groups attached to the nitrogen producing a tertiary nitro group and hence a neutral molecule, was found to be inactive as a coronary dilator, this suggests that the positive charge is essential for coronary dilator activity (Figure 11 & Table 2). This supports the finding, stated earlier, that the absence of the negatively charged (carboxylic acid) group from palmitoyl carnitine confers coronary dilator activity. In addition, Compound F containing a morpholinyl group was a very weak coronary dilator, and this compound is only weakly protonated at pH 7.4, and hence carries less positive charge than the quaternary ammonium compounds tested, which also indicates the importance of the positive charge on coronary dilator activity.

In the case of palmitoyl carnitine derivatives containing a quaternary nitrogen group (Compounds A-F) the larger the size of the groups attached to the quaternary nitrogen, the lower the positive charge on the nitrogen, due to the electron donating effect of the larger alkyl groups. The results obtained show that there is no simple relationship between the size of the positive charge and coronary dilator activity. The pattern observed was that the larger the size of the groups attached to the nitrogen, up to three ethyl groups, the greater the coronary dilator potency (Figure 11 & Table 2). Compound B (two methyls and one hydrogen) was significantly less potent and produced a significantly lower maximum dilator response than Compound A (three methyl groups) and Compound C (three ethyl groups) was significantly more potent than Compound A. However, the presence of three propyl groups attached to the nitrogen produces a compound with a potency not significantly

different from Compound A. Thus it appears that although the positive charge on the nitrogen is essential for coronary dilator activity, it is the conformation of the N terminus rather than the density of the positive charge on the quaternary nitrogen which confers coronary dilator potency. In the case of the classic calcium channel antagonists, a positive charge is not required for vasodilator activity, suggesting a different mechanism of action of palmitoyl carnitine derivatives. However, it has been shown that the binding of charged dihydropyridine calcium antagonists is unexpectedly stable, eg. amlodipine (Kass et al., 1988), due to a different mode of binding to the active site on the DHP receptor (Ferrante et al., 1990).

**1.3 Effect of C terminus substitution on coronary dilator potency.**

The group attached at the C terminus of the molecule appears to be less critical in determining the coronary dilator potency of the compound. All compounds tested which contained the same groups attached to the nitrogen (three methyls) were equieffective in terms of the maximum dilator response achieved (Table 3). However, Compound J which contains an overall double positive charge, due to the presence of two quaternary nitrogen groups, was significantly less potent than Compound A (Table 3 & Figure 12b), confirming the suggestion that the magnitude of the positive charge is not directly proportional to dilator potency. In addition, Compound G, containing a hydrogen in place of a C terminus group was significantly less potent than Compound A, which may relate to the fact that this compound showed a very large coronary constrictor component (Figures 13 & 14). The addition of an arginine group to the C terminus of the molecule (Compound M) produced a compound with significantly lower potency than Compound A, and not significantly different from Compound A in terms of maximum dilation achieved (Table 3 & Figure 12b). This suggests that Compound M does not elicit an additional coronary dilator activity by acting as a substrate for NO production. The nitroarginine equivalent (Compound N) was similarly lacking in potency. Interestingly, however, Compound N produced the most prolonged coronary dilation

of any compound tested, which may be related to the conformation of the molecule with a very long C terminus grouping.

In summary for all the structure-activity relationship studies, it is clear that an overall positive charge is essential for coronary dilator activity of palmitoyl carnitine derivatives, but the magnitude of the positive charge is not directly proportional to coronary dilator potency. The conformation of the groups at the N terminus of the molecule is critical to the potency of the compound, whilst changes in the C terminus produce smaller changes in coronary dilator potency, provided the grouping is uncharged. Additionally, most of the compounds tested also produced a transient coronary constrictor component to the response (see below). None of the compounds tested had any effect on cardiac contractility at the doses used. As small changes to the palmitoyl carnitine molecule have such a profound effect on coronary dilator activity, it seems likely that the mechanism of action is due to a specific interaction rather than a non specific detergent effect. The fact that a positive charge is essential for coronary dilator activity suggests that the mechanism may involve alteration of the surface charge on the membrane of the smooth muscle cells.

Compound G was selected for experiments investigating the mechanism underlying the coronary constrictor component of the response to the novel palmitoyl carnitine derivatives as this produced the greatest and most consistent coronary constrictor activity. The inhibition of the coronary dilator component of the response to compound G by the calcium antagonist verapamil is probably due to the fact that verapamil itself produced a large reduction in perfusion pressure (Figure 13), therefore allowing less scope for a coronary dilator response to Compound G. The inhibition of the coronary constrictor component of the response to Compound G by verapamil (Figures 13 & 14) suggests that it is either directly or indirectly affecting calcium entry through L-type calcium channels. Criddle et al.(1992; 1994) proposed that PIPi inhibits voltage gated calcium channels in the mesenteric vascular bed, and thus the coronary constrictor component of the response to these palmitoyl carnitine

derivatives may be via an opposing activation of voltage gated calcium channels. Verapamil has been shown to produce other effects in vascular smooth muscle apart from calcium antagonist activity, namely $\alpha_1$-adrenoceptor antagonist activity (Bhalla & Sharma, 1986; Müller & Noack, 1988). However, in smooth muscle membranes a concentration of 10$\mu$M verapamil was required to inhibit prazosin ($\alpha_1$-receptor) binding by 65-70% (Bhalla & Sharma, 1986), and therefore at the concentration used (100nM), verapamil is likely to exert mainly voltage gated calcium channel antagonist activity. Thus compound G, and hence other related palmitoyl carnitine derivatives, appear to be interacting with voltage gated calcium channels in some way, at least in eliciting the constrictor component to their response.

**2. Effect of high (110mM, 20mM) potassium solutions and TEA on the coronary dilator response to P1Pi.**

These experiments were performed in order to investigate an involvement of P1Pi with potassium channels in vascular smooth muscle, and to compare the coronary dilator activity of P1Pi with potassium channel opening coronary dilators and calcium antagonists, to elucidate further the mechanism for the vasodilator effect elicited by P1Pi and related compounds. The calcium channel blocker verapamil was used as a reference compound for these experiments as this has been shown by Hamilton et al. (1986) to be active in the presence of high (80mM) potassium-induced depolarisation, while the potassium channel opener cromakalim was inactive, in the rat portal vein.

A concentration of 110mM $K^+$ produced a depolarisation of the smooth muscle and the cardiac muscle which accounts for the increase in perfusion pressure, and the increase in resting tension and inhibition of contractility in the myocardium, shown in these experiments (Figure 15).

The coronary dilator responses to verapamil and nicardipine were not significantly affected in the presence of 110mM $K^+$ perfusate suggesting that calcium channel

blockers are unaffected by this perfusate. Papaverine is also considered to elicit a smooth muscle relaxation via inhibition of the calcium channel current (Iguchi et al., 1992), and although part of its relaxant effect has been attributed to phosphodiesterase inhibition, this is not the case in smooth muscle where vasodilation has been shown to occur in the absence of any increase in intracellular cAMP (Fujioka, 1984). The dilator response to this compound was also unaffected by 110mM $K^+$ perfusate, indicative of an action via calcium channel inhibition. The potassium channel opener lemakalim (Edwards and Weston 1990) is the active enantiomer of cromakalim, thought to act mainly via ATP-dependent potassium channels (Standen et al., 1989), although other types of potassium channel may also be involved in the blockade (Bray & Quast, 1991). The coronary dilator response to P1Pi, as well as to the potassium channel opener lemakalim, was completely inhibited by the presence of 110mM $K^+$ perfusate (Figure 16). This concentration of potassium depolarises the smooth muscle cells to -6mV, assuming the sarcolemma to be permeable to potassium ions only, calculated by the Nernst equation, shown in the 'Results' section. This greatly reduces the driving force for potassium efflux through potassium channels as the external potassium concentration is almost as high as the concentration within the smooth muscle cells. Hence, this prevents action of dilators which act via the opening of potassium channels and cell hyperpolarisation (Hamilton et al., 1986; Weir and Weston,1988). As the coronary dilator action of P1Pi, as well as the potassium channel opener lemakalim, was inhibited under 110mM $K^+$ conditions, this indicates a mode of action of P1Pi via the activation of potassium channels in smooth muscle. The fact that the response to salbutamol was also reduced in the presence of 110mM $K^+$ may be related to the fact that there was a tachyphylaxis shown to the coronary dilator effect of salbutamol in control preparations (Figure 17). However, the inhibition was even greater in the presence of 110mM $K^+$ perfusate, and there is evidence to suggest that ß-adrenergic agonists are able to increase $K^+$ currents (Bennett et al., 1986; Walsh & Kass, 1988; Wickenden & Ellis, 1991). Thus part of the vasodilator effect may be mediated by the opening of potassium channels in smooth muscle cells and this component can be inhibited in the presence of 110mM

potassium. The fact that the coronary dilator response to P1Pi in the presence of 110mM $K^+$ was different to that of verapamil and nicardipine is indicative of a different mechanism of action to that of the classic calcium channel antagonists. However, the results contradict those of Criddle et al. (1992; 1994), who showed that P1Pi was able to attenuate calcium-induced constrictions in the 110mM $K^+$ depolarised mesenteric vascular bed.

Experiments were also performed in the presence of 20mM $K^+$, as this concentration produces a degree of depolarisation within the vascular smooth muscle cells (from the Nernst equation, assuming sarcolemma to be permeable to potassium ions only, potential difference across sarcolemma = -51mV in the presence of 20mM $K^+$, see Results), but does not prevent the outward gradient for potassium following the activation of $K^+$ channels. Hamilton et al. (1986) showed that the potassium channel opener cromakalim could still inhibit calcium-induced constrictions in the rat portal vein in the presence of 20mM potassium, but not in the presence of 110mM potassium. The fact that P1Pi responses were significantly reduced but not abolished in the presence of 20mM $K^+$ perfusate (Figure 18) also points to a mode of action involving potassium channel opening, as the driving force for $K^+$ efflux will be reduced but not abolished.

Experiments were also carried out using TEA in order to follow up the hypothesis that P1Pi produces a coronary dilator response via the opening of $K^+$ channels. Criddle et al. (1994) found that glibenclamide had no effect on the vasodilator response to P1Pi. This agent is a blocker of ATP-dependent $K^+$ channels (Sturgess et al., 1988; Zunkler et al., 1988), although recent evidence has suggested an additional action, namely modulation of the dihydropyridine binding site of L-type calcium channels (Bian & Hermsmeyer, 1994). Lemakalim which was also inhibited in 110mM $K^+$ experiments acts via activation of ATP dependent $K^+$ channels. Therefore there is the possibility that another type of potassium channel is involved in the response to P1Pi, and so a less specific potassium channel blocker TEA (Cook

& Quast, 1990) was used to investigate this. TEA is able to block delayed (outward) rectifier $K^+$ channels, inward rectifier channels, high conductance $Ca^{2+}$ activated channels and sodium activated $K^+$ channels, and is also a weak blocker of the other classes of potassium channels (Cook, 1988). It has also been shown to exhibit 'non-specific' effects, ie. effects other than $K^+$ channel block at higher concentrations eg. block of muscarinic and nicotinic receptors. The fact that a high (10mM) concentration of TEA produced only a weak inhibition of the P1Pi response (Figure 19), together with the lack of effect of glibenclamide shown by Criddle et al. (1994) suggests that potassium channel activation is not the principal mode of action of P1Pi. This contradicts the results obtained with high (110mM) potassium solution. However it is important to note that in other perfused vascular preparations eg. the mesenteric vascular bed (Criddle et al., 1992; 1994) the actions of P1Pi were not prevented in 110mM $K^+$ depolarised preparations and therefore this also contradicts results obtained in the coronary vasculature and indicates differential effects in different vascular preparations.

## 3. Effects of P1Pi on responses to BAY K 8644, $PGF_{2\alpha}$, noradrenaline, methoxamine, caffeine and a low sodium perfusate in the isolated perfused rat heart.

### 3.1 The effect of P1Pi on BAY K 8644 responses in the isolated rat heart.

These experiments were originally performed in order to investigate the interaction of P1Pi with various coronary constrictor drugs in order to elucidate the mechanism for the coronary dilator action of P1Pi. In the case of experiments investigating the interaction with BAY K 8644, the interpretation of results was more simple as bolus doses of this compound produced a dose-dependent coronary constrictor effect with no concomitant effect on heart rate or developed tension (Figure 20). The lack of

effect of BAY K 8644 on developed tension was unexpected as this compound has been shown to produce a positive inotropic effect in cardiac muscle (Thomas et al., 1985; Böhm et al., 1985), but it could relate to the fact that the doses required to produce a large coronary constrictor effect were insufficient to produce any effect on the cardiac myocytes. The ability of P1Pi to attenuate the coronary constrictor action of the L-type calcium channel activator BAY K 8644 (Schramm et al., 1983; Thomas et al., 1985) in a concentration-dependent manner (Figure 21) indicates that P1Pi is able to exert an inhibitory action on L-type calcium channel activation in coronary smooth muscle. This supports the earlier findings of Criddle et. al. (1992;1994), who showed that P1Pi could inhibit calcium-induced contractions in perfused, potassium depolarised vascular beds. However, the data obtained from experiments using 110mM potassium depolarised rat hearts indicates that the action of P1Pi differs from the actions of classical calcium channel antagonists. It is possible that P1Pi may be exerting an inhibitory effect on BAY K 8644-induced contractions by affecting calcium-induced calcium release from the sarcoplasmic reticulum produced following calcium entry (Ito et al., 1991). This is not a likely explanation, however, since P1Pi, as a positively charged amphiphile, is unlikely to be able to cross the sarcolemma (Katz, 1992) and hence probably exerts its effect through a cell surface interaction. Additionally, the coronary constrictor effect induced by a low sodium perfusion, described later, was not affected by the presence of P1Pi, and this also has a calcium-induced calcium release component.

### 3.2 The effect of P1Pi on $PGF_{2\alpha}$ responses in the isolated rat heart.

$PGF_{2\alpha}$ has been shown to constrict coronary vessels (Nyborg & Mikkelsen, 1985; Schrör, 1993) via activation of PF prostaglandin receptors and possibly also thromboxane (TX) receptors (Schrör, 1993), and hence the interaction of P1Pi with this compound was also investigated, to test the specificity of the inhibitory action of P1Pi on BAY K 8644-induced coronary constriction. In this study, however, $PGF_{2\alpha}$

produced only a very weak coronary constrictor effect (Figure 27). This could be due to a low number of PF receptors in the coronary smooth muscle of this preparation or possibly due to $PGF_{2\alpha}$-induced release of vasodilator prostaglandins eg. prostacyclin (DeDeckere & Ten Hoor, 1980; Godfraind & Miller 1987) which would counteract the constrictor action of $PGF_{2\alpha}$. However, an unexpected positive inotropic effect was produced in response to $PGF_{2\alpha}$ which has seldom been reported previously, although Karmazyn et al. (1981) did report a positive inotropic effect of $PGF_{2\alpha}$ in the isolated rat heart. The second messenger system triggered following PF receptor stimulation is generally reported to be phospholipase C / $IP_3$ / DAG (Hatanaka et al., 1989; Coleman et al., 1994), although $PGF_{2\alpha}$ has also been shown to be a potent inhibitor of $Na^+$-$K^+$ATPase and thus could possibly exert a positive inotropic effect in a similar manner to that of the cardiac glycosides (Karmazyn et al., 1981). Although some prostaglandins have been shown to exert a positive inotropic effect via elevation of intracellular cAMP, this was found not to be the case for $PGF_{2\alpha}$ (Kangasaho et. al., 1978). A very low concentration (10nM) of P1Pi was found to completely inhibit the positive inotropic effect of $PGF_{2\alpha}$ while having no effect on basal contractility (Figures 27 & 28). As the precise mechanism for the $PGF_{2\alpha}$-induced positive inotropic effect remains unclear, this does not help elucidate the mechanism of action of P1Pi. However, it does indicate that P1Pi is able to gain access to the cardiomyocytes during coronary perfusion in the isolated rat heart preparation, and therefore this refutes the possibility that P1Pi does not affect basal contractility because of the inability to access the myocardium.

### 3.3 The effect of P1Pi on noradrenaline responses in the isolated rat heart.

In order to investigate further the inhibitory action of P1Pi on positive inotropic responses, the effect of P1Pi on the positive inotropic agent noradrenaline was investigated. This agent was found to produce more complex effects on the three parameters measured. In terms of effects on the coronary vasculature, the initial

coronary constrictor effect is probably mediated by a combination of $\alpha_1$ and $\alpha_2$ receptor stimulation (Heusch et al., 1984) plus a mechanical effect produced as a consequence of the positive inotropic action. The coronary dilator component is probably mediated mainly via $ß_2$ receptor activation (Gross & Feigl, 1975) and metabolic dilation, induced via the release of adenosine and dilator prostaglandins as a consequence of the large positive inotropic effect and increased workload of the heart (Zuberbuhler & Bohr, 1965). At higher doses of noradrenaline, this coronary dilator effect masked the coronary constrictor effect completely, thus making measurement of the effect of P1Pi on coronary constrictor responses to noradrenaline impossible (Figure 22). Although coronary vascular effects are markedly affected by changes in myocardial contractility, it has been shown previously that the reverse is not true ie. coronary perfusion pressure changes do not markedly affect cardiac contractility (Baydoun & Woodward, 1991), therefore studies using noradrenaline as an agonist concentrated mainly on examining the effect of P1Pi on the changes in myocardial contractility induced by this agent.

Noradrenaline produced a biphasic positive inotropic effect, and a monophasic positive chronotropic effect. The initial positive inotropic effect is probably mainly a consequence of the increase in heart rate, as sequentially increasing heart rate in the pacing experiments was found to produce an immediate increase in cardiac contractility (Figure 33), possibly via release of transmitters, eg. noradrenaline, due to stimulation of nerves in the myocardium. Therefore, only the second phase of the inotropic response was measured in these experiments. Positive inotropic and chronotropic responses to noradrenaline are mediated by stimulation of ß-adrenoceptors in the myocardium (Ahlquist, 1948), mainly of the $ß_1$ subtype (Molinoff, 1984). In the present studies, the $ß_1$-adrenoceptor antagonist atenolol virtually abolished both responses, indicating that the positive inotropic and chronotropic effects were mediated mainly via $ß_1$-adrenoceptor stimulation in this preparation. Noradrenaline has been shown to produce an α-adrenoceptor mediated positive inotropic response in the presence of ß blockade (Ask & Stene-Larsen, 1984) in rat

myocardium, but this effect was not seen in these studies. The $ß_1$-adrenoceptor mediated positive inotropic effect is due to G protein ($G_S$) stimulation of the adenylyl cyclase / cAMP / protein kinase A cascade followed by phosphorylation of L-type calcium channels which leads to an increase in calcium entry in cardiac myocytes (Sperelakis, 1988; Robishaw & Foster, 1989). Calcium channel activation in response to ß adrenergic agonists in cardiac myocytes has also been shown to occur via a direct G protein pathway independently of intracellular cAMP (Yatani et al., 1987; Yatani & Brown, 1989). However, this is probably not an important pathway physiologically, due to a basal regulation of calcium current by $G_S$, and increased activity above basal current requires phosphorylation (Hartzell et al., 1991; Harzell & Fischmeister, 1992). The chronotropic effect of noradrenaline is due to $ß_1$-adrenoceptor mediated activation of the pacemaker current, $I_f$, a hyperpolarisation activated $Na^+$ and $K^+$ inward current in the SA node, and both cAMP dependent phosphorylation and a direct effect of cAMP is thought to contribute to the activation of $I_f$ (DiFrancesco, 1993; DiFrancesco & Mangoni, 1994). The ability of P1Pi to inhibit the positive inotropic action of noradrenaline while having no effect on its chronotropic activity (Figures 23 & 24) was interesting, and the fact that atenolol inhibited both the positive inotropic and chronotropic effects of noradrenaline (Figures 23 & 24) indicates that P1Pi is not having a selective effect on the inotropic response simply due to poor perfusion of the drug to the SA node. The effect of P1Pi to supress positive inotropic responses to noradrenaline was shown in separate experiments to be reversible (Figure 32) and therefore the effect is not due to a non specific toxic effect of the compound, due to membrane disruption by the compound. As P1Pi did not affect either basal heart rate or the chronotropic action of noradrenaline, this indicates that it is not inhibiting pacemaker current, $ß_1$-adrenoceptors or cAMP production. This also implies that P1Pi is not acting in a similar manner to muscarinic agonists, or adenosine, to inhibit ß mediated positive inotropic responses via decreasing intracellular cAMP (Belardinelli & Isenberg, 1983; Robishaw & Foster, 1989; Fenton et al., 1991; Shyrock et al., 1993), as these two agents also inhibit the positive chronotropic response via a similar mechanism (DiFrancesco & Tromba, 1988).

These observations therefore point to an interaction with L-type calcium channels as the most likely site of action of P1Pi in inhibiting the inotropic response to noradrenaline. The lack of effect of P1Pi on basal cardiac contractility contrasts with its effect under noradrenaline stimulated conditions. Hence it appears that P1Pi is able to exert its inhibitory effect on phosphorylated L-type channels in cardiac myocytes. This possibility requires further investigation.

The direct coronary dilator effect of noradrenaline is mediated mainly via $ß_2$-adrenoceptors (Molinoff, 1984). However, in whole heart preparations, a metabolic vasodilation linked to an increased workload of the heart is also likely to contribute to the coronary dilator effect (Zuberbuhler & Bohr, 1965). The inhibition of the coronary dilator effect of noradrenaline by the $ß_1$-selective antagonist atenolol and by P1Pi (Figure 25) is most likely to be due to a reduction of metabolically induced vasodilation, via an inhibition of the positive inotropic effect induced by these two agents, and additionally, inhibition of the positive chronotropic effect in the case of atenolol. There is evidence for $ß_1$-adrenoceptor mediated coronary dilation in the rat heart (Nyborg & Michelsen, 1985) which may also account for some of the atenolol mediated reduction in the coronary dilator response. The metabolic dilation produced in response to an increase in heart rate is shown clearly in the pacing experiment shown in Figure 33.

The effects of the positively charged amphiphile polymyxin B, which has been shown to reduce ion fluxes in myocytes due to alteration of membrane surface charge (Burt et al., 1983), and palmitoyl carnitine, the parent molecule of P1Pi, on the positive inotropic response were investigated in order to establish the importance of the positive charge in the inhibitory effect of P1Pi. As polymyxin B was not able to significantly alter the positive inotropic response to noradrenaline (Figure 26), this indicates that the effect of P1Pi to reduce these responses is not solely due to the fact that it is a positively charged amphiphilic substance. However, as palmitoyl carnitine was also ineffective, the positive charge of P1Pi is important in the inhibitory effect.

### 3.4 Effect of P1Pi on methoxamine responses in the isolated rat heart.

Although noradrenaline is an agonist at both α and ß adrenoceptors, in these studies its actions appeared to be mediated mainly via the activation of ß receptors within the myocardium. Also, the dilator effect of noradrenaline masked the α mediated constriction in the coronary vasculature. Therefore, the α selective agonist methoxamine was used to investigate the effect of P1Pi on α mediated responses, in both the myocardium and coronary vessels. The effect of P1Pi on responses to a ß selective agonist eg. isoprenaline was not used, as preliminary experiments showed this compound to depress basal coronary contractility following the positive inotropic effect to such an extent that dose response curves could not be constructed. Methoxamine produced a variable effect on perfusion pressure, a monophasic positive inotropic effect and no concomitant effect on heart rate. Stimulation of α-adrenoceptors is able to produce vasoconstriction (Molinoff, 1984), however the lack of a consistent coronary constrictor effect in this preparation may be due to an opposing metabolic vasodilation, similar to that described for noradrenaline (see Discussion, section 3.3), although a marked coronary dilator effect was not observed in response to methoxamine. The fact that the positive inotropic response was monophasic, unlike noradrenaline which showed a biphasic positive inotropic response may reflect the absence of any increase in heart rate shown in response to methoxamine, and this is likely to be responsible for the initial positive inotropic response produced by noradrenaline. Methoxamine is believed to produce a positive inotropic response in the absence of any increase in the calcium inward current in isolated cardiomyocytes (Hartmann et al., 1988; Terzic et al., 1991; Puceat et al., 1992), although some studies have reported an increase in slow inward current in response to α-adrenoceptor agonists, independent of cAMP (Lindemann, 1986; Otani et al., 1988; Fedida et al., 1993), via protein kinase C induced phosphorylation of L-type calcium channels in the myocytes (Lacerda et al., 1988). Michel et al.,(1994) have suggested that the calcium influx is produced only by stimulation of $\alpha_{1A}$

receptors and not $\alpha_{1B}$ receptors, and $\alpha_{1A}$ receptors were found to function in the rat ventricle only in the presence of $\alpha_{1B}$ receptor blockade; this suggests that calcium entry is not an important mechanism in the effect of $\alpha_1$-adrenoceptor agonists physiologically. Alpha 1-adrenoceptor agonists are believed to produce a positive inotropic effect via myofilament sensitization (Puceat et al., 1992), ie. in a manner independent of an increase in intracellular calcium. The effector mechanism following $\alpha_1$-adrenoceptor stimulation in myocytes is G protein activated phospholipase C induced phosphatidylinositol (1,4,5) bisphosphate hydrolysis yielding inositol (1,4,5) triphosphate and diacyl glycerol (Brown et al., 1985; Woodcock et al., 1987; Otani et al., 1988; Fedida et al., 1993). Diacyl glycerol mediated activation of protein kinase C is believed to enhance myofilament responsiveness to calcium via phosphorylation of the $Na^+$-$H^+$exchanger producing an intracellular alkalinisation (Terzic et al., 1991; Fedida et al., 1993; Gambassi et al., 1992) and direct phosphorylation of contractile proteins (Puceat et al., 1990). The role of inositol triphosphate ($IP_3$), which can produce a release of calcium from the sarcoplasmic reticulum, in the inotropic effect believed to be unimportant in myocardial cells (Woodcock et al., 1987; Langer, 1992). The fact that P1Pi inhibited $\alpha_1$-adrenoceptor agonist-induced increases in cardiac contractility whilst having no effect on basal contractility (Figure 29), and that the $\alpha_1$-mediated positive inotropic effect occurs in the absence of any increase in intracellular calcium suggests that P1Pi is not acting via a supression of calcium influx through L-type channels in these experiments, as was suggested as the mechanism for inhibition of ß-mediated positive inotropic responses in previous experiments. This suggests that P1Pi can exert an inhibitory action on second messenger systems other than the adenyl cyclase / cAMP / protein kinase A cascade and L-type calcium channel activation. In the case of supression of $\alpha_1$ mediated positive inotropic responses (and possibly also $PGF_{2\alpha}$-mediated positive inotropic responses, which may involve a similar mechanism to methoxamine responses as $PIP_2$ hydrolysis is a known effector mechanism for $PGF_{2\alpha}$ (Hatanaka et al., 1989; Coleman et al., 1994)), a possible mechanism of action is via inhibition of protein kinase C. This action has been

shown for other cationic amphiphiles (Epand & Lester, 1990; Bottega & Epand, 1992) eg. polymyxin B (Raynor et al., 1991), as well as for zwitterionic membrane stabilizers (Bottega & Epand, 1992) eg. the parent molecule palmitoyl carnitine (Wise et al., 1982; Epand & Lester, 1990). Bottega & Epand (1992) also suggest that compounds which are both cationic, particularly quaternary ammonium compounds, and membrane stabilizers would be the most potent inhibitors of protein kinase C, which would make P1Pi a likely candidate for this action. These results point to another mechanism of action, possibly by affecting receptor-second messenger coupling, or possibly by inhibiting specific protein kinases. The fact that polymyxin B is also an inhibitor of protein kinase C, but did not exert the same inhibitory effect as P1Pi on inotropic responses suggests that P1Pi has a different mode of action. However, other reports have shown that polymyxin B is required in higher concentrations than were used in this study in order to inhibit protein kinase C activation (Raynor et al., 1991), with an $EC_{50}$ of approximately 20$\mu$M. The concentration of 1$\mu$M was chosen for this study in order to investigate the effects of the molecule due to charge, and this concentration would produce an even greater increase in surface positive charge than the concentration of P1Pi (10nM) which produced an inhibitory effect on positive inotropic responses.

### 3.5 Effect of P1Pi on caffeine responses in the isolated rat heart.

Caffeine was required in much higher doses to elicit effects in the isolated perfused rat heart than other agents used. This is because it does not act via activation of specific membrane receptors like the other agents studied. It produced a transient variable positive inotropic effect and a transient coronary constrictor effect followed by a longer lasting coronary dilator component (Figure 30). Caffeine is a phosphodiesterase inhibitor (Butcher & Sutherland, 1962), and this is likely to contribute to both the positive inotropic (Bers, 1985; Rousseau & Meissner, 1989) and the coronary dilator (Van Breemen & Saida, 1989) effect of caffeine, through an

increase in intracellular cAMP levels in cardiac and coronary smooth muscle, if basal levels of cAMP are present. Caffeine also increases calcium release from the sarcoplasmic reticulum, via stimulation of sarcoplasmic reticulum ryanodine receptors (Van Breemen & Saida, 1989; Watanabe et al., 1992) and this is probably the mechanism for the coronary constrictor action produced by caffeine. As P1Pi failed to inhibit the coronary constrictor effect of caffeine (Figure 31), this indicates that it is not acting via inhibition of ryanodine receptors of the sarcoplasmic reticulum to inhibit calcium release.

### 3.6 The effect of P1Pi on the response to low sodium perfusion in the isolated rat heart.

Low sodium perfusion produced complex effects in the isolated perfused rat heart preparation (Figure 36). The initial coronary constrictor effect and the positive inotropic effect are likely to be due to increase in intracellular calcium produced by perturbation of the $Na^{+}$-$Ca^{2+}$ exchange process, shown to occur in both vascular smooth muscle (Batlle et al., 1991) and cardiac muscle (Bridge et al., 1990; Philipson, 1990). Other cationic amphiphiles have been shown to inhibit this exchange process (Philipson et al., 1985; Hilgemann & Collins, 1992) through an effect on membrane surface charge, possibly to oppose stimulation by negatively charged phospholipids in the sarcolemma (Collins & Hilgemann, 1993). An inhibition has also been reported for the parent compound palmitoyl carnitine (Philipson & Nishimoto, 1982; Lamers et al., 1984). However, the lack of effect of P1Pi on the positive inotropic or the coronary constrictor responses shows that under the conditions studied, it is not having any significant effect on $Na^{+}$-$Ca^{2+}$ exchange across cardiac muscle and coronary smooth muscle cells. The lack of effect is not due to the fact that we are seeing a supramaximal effect of low sodium as lowering the sodium chloride concentration below 59mM, as shown in the concentration-effect curve to sodium chloride in Figure 34 and Figure 35, results in even greater positive inotropic and coronary constrictor responses. As with the coronary

constrictor response produced by BAY K 8644, calcium-induced calcium release from the sarcoplasmic reticulum will be involved in the positive inotropic (Rich et al., 1988; Bers, 1985; Leblanc & Hume, 1990; Kohmoto et al., 1994) and coronary constrictor (Ito et al., 1991) effects of low sodium perfusion. As P1Pi had no effect on either of these parameters, this provides evidence to suggest that P1Pi is not affecting calcium-induced calcium release, in agreement with the results obtained for caffeine in the coronary vasculature. Low sodium perfusion also produced a decrease in heart rate (bradycardia) due to hyperpolarisation of the pacemaker cells. Under normal conditions, $Na^{+}$-$Ca^{2+}$ exchange is electrogenic, three sodium ions entering the cell being exchanged for one calcium being extruded (Philipson & Nishimoto, 1980). Therefore, a decrease in this exchange will produce a more negative membrane potential. The lack of effect of P1Pi on this bradycardia is further evidence to suggest that P1Pi does not inhibit $Na^{+}$-$Ca^{2+}$ exchange or the pacemaker current $I_f$. The secondary coronary dilator component of the response to low sodium occurs as a consequence of the positive inotropic effect, as previously described for noradrenaline, ie. release of vasodilator metabolites linked to the increased workload of the heart (Zuberbuhler and Bohr, 1965). The inhibition of these dilator responses by P1Pi is more difficult to explain but could possibly be inhibiting the release of vasodilator metabolites, or the coupling of these metabolites' receptors to their respective second messenger systems. The latter explanation would be in agreement with the inhibition by P1Pi of agonist-induced positive inotropic responses in the myocardium.

### 3.7 Summary of the effect of P1Pi on responses to various agonists in the isolated rat heart.

In summary, in vascular smooth muscle, P1Pi was found to inhibit the coronary constrictor responses to the calcium channel activator BAY K 8644, but not responses to a low sodium perfusion or caffeine. In the myocardium, P1Pi was found to have no effect on basal contractility, but inhibited the positive inotropic responses to $PGF_{2\alpha}$,

noradrenaline and methoxamine, but not the response to low sodium perfusion. In addition, a high (110mM) potassium perfusion inhibited P1Pi-induced coronary dilation, but not verapamil-induced dilation.

The fact that both caffeine-induced coronary constriction, and low sodium mediated coronary constriction and increase in myocardial contractility, all of which have a calcium release component (see above), were unaffected suggests that the mechanism(s) of action of P1Pi does not include modulation of sarcoplasmic reticulum ryanodine receptors. This can also be considered unlikely on the grounds that as a cationic compound, P1Pi is unlikely to be able to cross the sarcolemma (Katz, 1992), and therefore it probably exerts its action on the outer sarcolemmal surface. The ability of P1Pi to inhibit BAY K 8644-induced coronary constriction indicates that P1Pi is able to inhibit L-type $Ca^{2+}$ channel activation, and this is supported by its ability to inhibit ß-mediated positive inotropic responses within the myocardium. Post et. al. (1991) described similar effects for the positively charged amphiphile dodecyl trimethylammonium (DDTMA), which inhibited the calcium current through L-type channels in myocytes, an effect attributed to a change in the surface charge and hence transmembrane potential. However, the inhibition of methoxamine and $PGF_{2\alpha}$-induced positive inotropic responses does not fit this theory. The mechanisms by which noradrenaline, methoxamine and possibly $PGF_{2\alpha}$ exert a positive inotropic effect involves phosphorylation processes, either by protein kinase A or protein kinase C. Epand (1987) suggested that substrate phosphorylation by protein kinase C can be inhibited by positively charged compounds, and it is possible that protein kinase A mediated phosphorylation may be inhibited in the same manner. The ability of second messenger mediated protein phosphorylation to activate ion channels or ion transport systems has been suggested to be due to the incorporation of an anionic (phosphate) group on the membrane surface, thus decreasing the potential across the sarcolemma and increasing membrane excitability (Katz, 1992), and cations may therefore counter this effect. It is possible that following incorporation of P1Pi into the outer sarcolemmal leaflet, by way of its fatty acid chain, close to the membrane proteins, it

may counteract the effects of protein phosphorylation by means of the positively charged quaternary nitrogen group. In the case of ß mediated positive inotropic responses ie. in response to noradrenaline-induced stimulation, the phosphorylation sites are on the $\alpha_1$ and ß subunits of the L-type calcium channel (Catterall & Striessnig, 1992), and P1Pi could be affecting calcium currents by countering the effects of phosphorylation at this site. Pacemaker activity in the SA node cells of the myocardium is regulated by the hyperpolarisation activated current, $I_f$, carried by $Na^+$ and $K^+$ ions, although other current (eg. calcium current through T-type channels and cAMP regulated chloride current) are also believed to be involved (DiFranceso, 1993). The main current responsible for pacemaker activity is $I_f$, and the fact that increases in heart rate were unaffected by P1Pi also agrees with this hypothesis, as cAMP is able to control the activity of this current directly, a mechanism which is now considered to be more important than via protein kinase A induced phosphorylation of the channel (DiFrancesco, 1993; DiFrancesco & Mangoni, 1994). A direct G protein activation of $I_f$ in pacemaker cells (Yatani et al., 1990) has also been suggested. The main mechanisms of activation of $I_f$ are therefore largely independent of phosphorylation, which may explain the lack of effect of P1Pi on noradrenaline-induced increases in heart rate and hence the pacemaker current. Doolan & Keenan (1994) reported an inhibitory action of fatty acids eg. arachidonic acid on protein kinase A-induced phosphorylation, and P1Pi may be acting similarly. In the case of the $\alpha_1$-adrenoceptor mediated methoxamine responses, P1Pi may be modulating sarcolemmal protein kinase C activity. Therefore in this case inhibition of the protein kinase C phosphorylation-activated $Na^+$-$H^+$ exchanger is a possible mechanism of action for P1Pi-induced inhibition of positive inotropic responses in these experiments. The main direct effects of protein kinase C are in the sarcolemma, as it requires the correct lipid environment for activation ie. the presence of negatively charged membrane phospholipids eg. phosphatidyl serine, phosphatidylinositol, phoshatidic acid (Oishi et al., 1988; Epand & Lester, 1990: Sando et al., 1992). Effects of protein kinase C to activate myofilaments could involve the activation of a kinase cascade, as alone it is

insufficient for activation of the myofilaments (Puceat et al., 1990), and therefore a sarcolemmal effect of P1Pi could also interfere with this cascade. The fact that protein kinase C requires an association with negative amphiphiles (phospholipids) for activity has led to the proposal that positively charged amphiphiles exert their inhibitory effect by opposing this interaction, following incorporation into the sarcolemma (Epand & Lester, 1990), and P1Pi may also be acting in this way.

Another possible explanation for the inhibition by P1Pi of the receptor mediated positive inotropic responses but not the low sodium perfusion mediated positive inotropic response is a direct G protein interaction, which has previously been shown with other cationic amphiphiles, eg. substance P (Mousli et al., 1990). However, very high concentrations are required for this charge dependent effect, and the mediators are generally doubly charged and therefore it seems unlikely that P1Pi is acting through a direct G protein mechanism. In addition, the fact that P1Pi does not affect the pacemaker current $I_f$, which is also mediated via a G protein pathway (DiFrancesco, 1993), also indicates that P1Pi is unlikely to act via a direct interaction with G proteins.

The fact that P1Pi produces a coronary dilation with no effect on basal contractility (this study; Criddle et. al., 1990) suggests a different mode of action within vascular smooth muscle to that in the myocardium. In addition, the ability of P1Pi to antagonise BAY K 8644-induced contractions suggests an ability to affect calcium channel currents independent of phosphorylation in smooth muscle. However the possible interaction of P1Pi with membrane surface charges could also explain these effects. A more negative transmembrane potential close to the calcium channels in smooth muscle, which could occur following binding of positively charged P1Pi, may affect the voltage gating of the channel and hence opening of the calcium channel (Green & Andersen, 1991; Latorre et al., 1992). The fact that basal myocardial contractility is unaffected may be due to the relative contribution of calcium entry and release from the sarcoplasmic reticulum on basal contractility in this cell type. In rat

myocytes, calcium entry through L-type calcium channels is insufficient to activate myofilaments directly, but a small influx of calcium is amplified through calcium-induced calcium release from the sarcoplasmic reticulum, which then activates the contractile filaments (Fabiato, 1983; Rich et al., 1988; Bers, 1985; Langer, 1992). This initial calcium entry which triggers calcium release may also be mediated by sodium influx, and subsequent calcium entry via the $Na^+$-$Ca^{2+}$ exchanger (Leblanc & Hume, 1990; Kohmoto et al., 1994), as well as entry through L-type calcium channels. In terms of vascular smooth muscle, the earlier findings that P1Pi only exerted a relaxant effect in the microvasculature and not in conduit vessels (Criddle et al., 1992) is further evidence for an effect only on the sarcolemmal surface, to modify ion currents via a charge effect, as agonist-induced release of sarcoplasmic reticulum calcium is more prominent in the large conduit arteries, but is diminished in smaller arteries, where calcium entry is more important (Goldman et al., 1988). Thus the lack of effect of P1Pi on conduit vessels may be due to the same reason as the lack of effect on basal contractility within the myocardium.

The inhibition of the coronary dilator response to P1Pi by high potassium perfusion may also be explained by the membrane charge effect. The high degree of depolarisation of smooth muscle cells achieved by 110mM $K^+$ perfusion may counter the increase in negativity of the transmembrane potential which could be responsible for the dilator action of the positively charged amphiphile P1Pi, thus preventing the coronary dilator response. It appears unlikely that P1Pi is exerting a direct effect on potassium channels as neither glibenclamide (Criddle et al., 1994) nor TEA (this study) abolished coronary dilator responses to P1Pi. However, it could be producing a similar effect to potassium channel openers, through increasing the positive charge on the outer sarcolemmal surface and thus producing a more negative transmembrane potential, an action which has been described for other cationic amphiphiles (Post et al., 1991). Potassium channel openers, by hyperpolarising the cell, also increase the transmembrane potential, thus reducing membrane excitability and rendering the cell more resistant to activation by agonists (Quast et al., 1994). In previous studies in the

mesenteric vascular bed of the rat (Criddle et al., 1992; 1994), the finding that P1Pi was still able to prevent calcium-induced vasoconstrictor responses may be due to the fact that higher concentrations of P1Pi were used to counter the depolarisation. There may also be differences in the resting membrane potential in the two preparations. Chappell et al. (1990) found that in pulmonary vessels, much higher external potassium concentrations were required to inhibit 1$\mu$M lemakalim mediated relaxation in small pulmonary vessels than in large vessels, and additionally, a low (0.1$\mu$M) concentration of lemakalim was able to maximally relax the low potassium-depolarised small vessel while having no effect in the large vessel. These effects were attributed to differences in resting membrane potential.

### 3.8 Comparison of the effects of palmitoyl carnitine with those of P1Pi in these studies.

The parent molecule, palmitoyl carnitine did not share the properties of the isopropyl ester derivative in isolated perfused heart studies. However, it has been shown in erythrocyte electrophoretic mobility (EPM) studies that palmitoyl carnitine itself confers a positive charge on the membrane surface (Meszaros et. al., 1988) and this has been suggested as a mechanism of many of the sarcolemmal effects of higher concentrations ($>10\mu$M) of palmitoyl carnitine eg. inhibition of $Na^{+}$-$Ca^{2+}$ exchange (Philipson & Nishimoto, 1982; Lamers et al., 1984) and inhibition of excitatory ion currents (Inoue & Pappano, 1983; Meszaros et al., 1988). In the present studies the concentrations of P1Pi and palmitoyl carnitine used were the same (10nM), and higher concentrations of palmitoyl carnitine than of P1Pi may be required in order to produce an increase in positive charge on the sarcolemmal surface, which may explain the lack of effect of palmitoyl carnitine on positive inotropic responses in the present experiments. In addition, both the lack of effect of palmitoyl carnitine on the positive inotropic response and the opposite effect of palmitoyl carnitine to that of P1Pi in the coronary vasculature ie. coronary constriction rather than dilation may be due to other

effects of palmitoyl carnitine on the positive inotropic response and the opposite effect of palmitoyl carnitine to that of P1Pi in the coronary vasculature ie. coronary constriction rather than dilation may be due to other effects of palmitoyl carnitine counteracting the inhibition of calcium currents. For example, Wu & Corr (1992) using direct electrophysiological measurements within cardiac myocytes showed that palmitoyl carnitine decreased calcium currents but produced an increase in intracellular calcium secondary to an increase in intracellular sodium via a slowed inactivation of $Na^+$ inward current (Wu & Corr, 1994), and hence calcium entry through reversed $Na^+$-$Ca^{2+}$ exchange. This may also trigger calcium-induced calcium release from the sarcoplasmic reticulum (Leblanc & Hume, 1990), as the release channel has been shown to be situated close to the exchanger (Moore et al., 1993), and therefore this may help explain the actions of palmitoyl carnitine to increase intracellular calcium. An increase in calcium release from the sarcoplasmic reticulum induced by palmitoyl carnitine has been shown in both skeletal (El-Hayek et al., 1993) and cardiac (Meszaros & Pappano, 1990) muscle. Studies showing palmitoyl carnitine to act as a calcium channel activator in smooth and cardiac muscle (Patmore et al., 1989; Spedding & Mir, 1987) were mostly indirect studies, and an increase in calcium may occur secondary to other cellular effects of palmitoyl carnitine. The effect of palmitoyl carnitine in vascular endothelial cells to inhibit agonist-induced release of nitric oxide (via the PLC / $IP_3$ / DAG pathway) has been ascribed to supression of receptor mediated signal transduction following incorporation of the amphiphile into the sarcolemma (Inoue et al., 1994), since an inhibition of nitric oxide release mediated by several different agonists is produced eg. bradykinin-induced release (Inoue et al., 1994), ATP and carbachol mediated release (Dainty et al., 1990). In addition, palmitoyl carnitine has been shown to uncouple ß-adrenoceptor mediated transduction pathways in the myocardium (Meszaros & Levai, 1992; Abe et al., 1984). These effects reflect P1Pi mediated suppression of agonist-induced positive inotropic responses, and a similar mechanism may be responsible, although at the concentrations used in the present studies, no effect on positive inotropic responses was produced by palmitoyl carnitine itself.

**4. Effect of palmitoyl carnitine and P1Pi on ischaemia / reperfusion in the isolated rat heart.**

As P1Pi had been shown to inhibit agonist-induced positive inotropic responses, produced by both α- and ß-adrenoceptor agonists and by $PGF_{2\alpha}$, its effects on simulated ischaemia / reperfusion were also investigated, as this has been shown to induce both catecholamine and prostaglandin release (Ceremuzynski et. al., 1969; Berger et al., 1976; Van der Vusse & Reneman, 1985). Additionally, as P1Pi has been shown during the course of these experiments to have characteristics in common with both calcium channel antagonists and potassium channel openers, P1Pi may be expected to exert protective effects in a model of global ischaemia / reperfusion. Many classes of calcium channel antagonist have been shown previously to exert protective effects from global ischaemic injury in isolated hearts (Watts et al., 1986), possibly via depression of cardiac contractility and conservation of high energy phosphates, which are depleted in the ischaemic myocardium (Haworth et al., 1981), the result of which is impaired calcium homeostasis due to a run down of membrane ion pumps in myocytes (Van der Vusse & Reneman, 1985). An excessive increase in calcium entry through $Na^+$-$Ca^{2+}$ exchange is thought to occur upon reperfusion, due to an increase in intracellular sodium following both a rundown of the sodium pump and stimulation of the $Na^+$-$H^+$ exchanger as a consequence of ischaemia, and this is believed to contribute to the impairment of contractility upon reperfusion (Tani & Neely, 1989). Potassium channel openers eg. cromakalim have been shown to be protective, through both increasing coronary flow upon reperfusion, reducing myocardial calcium levels and conservation of myocardial ATP levels (Weston & Edwards, 1992; Gross & Auchampach, 1992; Grover, 1994). Specific calcium 'leak' channels, activated following ATP depletion, are also implicated in ischaemia / reperfusion injury and cationic amphiphiles have been shown to have inhibitory effects on these channels (Clague et. al., 1993; Clague & Langer, 1994). Therefore P1Pi, another cationic amphiphile, may confer protection via this mechanism.

In these experiments, each period of global ischaemia was limited to 20 minutes, so that recovery of myocardial function occurred on reperfusion, as 40 minutes or more of ischaemia leads to irreversible myocardial cell injury due to calcium overload (Chien & Engler 1990). However there was a slight attenuation of basal contractility shown during each period of reperfusion, indicating that some damage had occurred. Neither P1Pi nor palmitoyl carnitine were able to significantly improve the recovery of developed tension on reperfusion, which has been shown to be restored by the administration of calcium antagonists (Watts et al., 1986; Chien & Engler 1990), suggesting that calcium entry through L-type calcium channels is involved in reversible injury to the myocardium. This may be related to the fact that calcium antagonists protect the heart via a decrease in basal myocardial contractility which conserves high energy phosphates (Watts et al., 1986; Haworth et al., 1981) and P1Pi does not affect basal contractility. However, potassium channel openers have also been shown to enhance recovery of contractility on reperfusion following global ischaemia (Grover et al., 1989; Gross & Auchampach, 1992), probably through preservation of cellular ATP levels, in the absence of a reduction in myocardial contractility. The ischaemic contracture produced during the first 20 minutes of ischaemia, believed to be associated with loss of tissue ATP (Haworth et al., 1981) was slightly reduced by the higher (100nM) concentration of P1Pi used (Figures 37 & 38). This ischaemic contracture occurs through an accumulation of intracellular calcium, the mechanism for which may involve increased calcium entry through L-type calcium channels, $Na^{+}$-$Ca^{2+}$ exchange or calcium 'leak' channels or decreased calcium extrusion via sarcolemmal $Ca^{2+}$-ATPase(Haworth et al., 1981; Tani & Neely, 1989; Clague et al., 1993). At higher concentrations, P1Pi may be able to inhibit calcium entry under conditions of ischaemia possibly by inhibition of calcium entry through L-type calcium channels or inhibition of the calcium 'leak' channels, shown to be inhibited by other positively charged amphiphiles (see above). In addition, P1Pi was able to significantly inhibit the slowly developing coronary constriction which occurred following reperfusion (Figure 37 & 40). This could be due to the release of vasoconstrictor prostaglandins and catecholamines

(Ceremuzynski et al., 1969; Berger et al., 1976), and P1Pi might be inhibiting the release of these mediators or the coupling of these mediators to their respective second messenger systems, similarly to that suggested for the P1Pi mediated inhibition of agonist-induced positive inotropic effects. The lack of any significant effect of the parent compound palmitoyl carnitine on these responses indicates that it is a specific effect of the ester derivative. Further studies are required, however, to establish which mediators are involved in the coronary constrictor effect and also to investigate whether or not other coronary dilators eg. calcium antagonists, are able to produce the same effect in this preparation.

**5. Effect of P1Pi in the isolated, stimulated left atrium of the rat.**

These studies were performed to investigate whether P1Pi can exert the same inhibitory effect in isolated cardiac muscle preparations, and to separate coronary vascular effects from the effects on cardiac muscle. Noradrenaline added cumulatively to the organ bath produced a cumulative increase in developed tension, due to $ß_1$-adrenoceptor stimulation and subsequent increase in calcium influx through calcium channels, following an increase in intracellular cAMP, in a similar way to that described in the perfused heart. An additional $\alpha$-adrenoceptor mediated positive inotropic effect may also have contributed to the response (See section 3, Discussion). P1Pi alone, up to 1$\mu$M had no effect on basal contractility in response to electrical stimulation, and did not affect the noradrenaline-induced positive inotropic response in this preparation, nor the twitch length in the presence of noradrenaline (Figures 41, 42 & 43). There are two possible explanations for the difference in the effect of P1Pi in the isolated stimulated left atrium, compared with the perfused heart preparation. Firstly, P1Pi present in the bathing solution is not able to gain access in sufficient concentration to exert an inhibitory effect in the stimulated atrium, and perfusion via the coronary vessels is a far more efficient mechanism for delivery of the compound to individual cardiac myocytes. Secondly,

P1Pi may be acting indirectly on cardiac muscle via the release of inhibitory substances from the coronary vasculature. In order to investigate these possibilities further, studies were performed in isolated myocytes, monitoring intracellular calcium levels by fluorimetric measurement.

**6. The effect of P1Pi on calcium fluxes in isolated perfused rat myocytes by fluorimetric analysis.**

In these experiments, a single myocyte stimulated at 1Hz, perfused at 2ml/min with 1mM $Ca^{2+}$ physiological salt solution was found to produce transient increases in the 340/380 fura-2 signal ratio which synchronised with each electrical stimulation. As explained in the results section, the 340/380 ratio is directly proportional to intracellular calcium concentration and therefore these increases represent intracellular calcium transients, brought about by depolarisation of the myocyte with each electrical stimulus, and subsequent calcium entry through L-type calcium channels. The addition of P1Pi to the perfusion medium at a concentration of 100nM produced no effect on the size of these basal calcium transients. This is in agreement with the results obtained in the isolated perfused rat heart in which P1Pi had no effect on basal contractility. However, P1Pi did appear to increase the 'noise' of the measured calcium transients, producing less smooth increases in the 340/380 fura-2 ratio signal in response to electrical stimulation (Figure 48a). This effect is less easily explained, but may involve the interaction of P1Pi with ion currents in the myocyte. This possibility requires further study. Noradrenaline was found to produce a slowly developing but sustained increase in calcium transients in individual myocytes (Figure 47a). A concentration-dependent response could not be produced to noradrenaline, due to the large variability in the responses to noradrenaline in different individual myocytes. This is not surprising as the entire population of ventricular myocytes was harvested from each heart, and therefore individual cells under investigation may have been from any region of the left or right ventricle, and

are likely to vary in their adrenoceptor numbers and degree of response to electrical stimulation. Future studies could improve on this by harvesting cells from just one area of the heart eg. right ventricular cells. Despite variability between experiments, the average increase in the calcium transients was comparable to that obtained by other groups using similar concentrations of isoprenaline (10nM-1$\mu$M) ie. 150-200 % of control transients (Marsh, 1990; Hancox et al., 1994; Fenton et al., 1991). In addition to increasing the size of the calcium transients, noradrenaline also decreased the duration of the calcium transients (Figure 47b). This parallels the well established lusitropic action of ß-adrenergic agonists, to shorten the time course of each contraction (Tsien, 1977). This effect is due to the ability of noradrenaline to enhance re-uptake of calcium into the sarcoplasmic reticulum, by cAMP dependent protein kinase A-induced phosphorylation of phospholamban, which stimulates the SR $Ca^{2+}$-ATPase pump (Kranias, 1985; Davies et al., 1993), and also protein kinase A-mediated phosphorylation of troponin C which decreases the sensitivity of the myofilaments to calcium and increases the rate of cross bridge detachments from actin (Puceat et al., 1990; Strang et al., 1994). The complete inhibition of the noradrenaline-induced increase in the size of the calcium transients by atenolol (Figure 49) indicates that ß receptor stimulation is responsible for these increases, via increased cAMP accumulation within the cell and a subsequent phosphorylation of L-type calcium channels by cAMP dependent protein kinase A. P1Pi (100nM) also completely inhibited the noradrenaline-induced increase in calcium transients (Figure 48a), probably via the same mechanism as in the isolated perfused heart (see above). This indicates that P1Pi is having a direct effect on the myocytes, and the inhibitory effect is not due to the release of mediator(s) from the coronary vasculature. Therefore, the lack of any effect of P1Pi in the isolated stimulated left atrium is probably due to difficulties in the access of the molecule to individual myocytes. The lack of effect of P1Pi on the noradrenaline-induced shortening of the duration of the calcium transient (Figure 48b) is indicative of the membrane surface effect of P1Pi, and the inability to cross the sarcolemma to modulate intracellular processes eg. sarcoplasmic reticulum $Ca^{2+}$-ATPase. However, as indicated in the results

section, P1Pi itself appeared to decrease the duration of the calcium transients which may imply a modulation of basal calcium within myocytes eg. a reduction in the action potential duration through an inhibition of currents through L-type calcium channels in a similar manner to that proposed in smooth muscle (Criddle et al., 1994). This is an effect which cannot be observed in whole heart preparations.

The effects observed in response to caffeine in isolated myocytes ie. to increase the duration of the calcium transients, while having no effect on the amplitude (Figure 50) may be explained by the fact that calcium release from the ryanodine sensitive calcium release channel of the sarcoplasmic reticulum, produced in response to caffeine (Rousseau & Meissner, 1989), occurs close to the sarcolemmal $Na^{+}$-$Ca^{2+}$ exchanger, and calcium efflux via this exchanger may limit the peak of the $Ca^{2+}$ transient in response to caffeine (Bassani et al., 1992). This may also explain the lack of effect of caffeine on myocardial contractility in the isolated perfused rat heart. The increase in duration of the calcium transient in the presence of caffeine may be due to an inhibition of reuptake of calcium, another effect produced by caffeine (Bers, 1985). The lack of any effect of P1Pi on the action of caffeine is further evidence to suggest that P1Pi does not affect calcium release from the sarcoplasmic reticulum.

As verapamil was able to block the calcium transients in response to electrical stimulation, this indicates that calcium entry is the initial trigger for depolarisation-induced calcium transients within myocytes.

## Conclusions

These studies have investigated the actions of some novel palmitoyl carnitine derivatives, for example the isopropyl ester of palmitoyl carnitine (P1Pi) in coronary smooth muscle and cardiac muscle. The original aim was to investigate the mechanism of the coronary dilator response of these novel compounds. The structure-activity relationship studies have shown that the positive charge on the molecule is essential for coronary dilator activity, although the magnitude of the positive charge is not directly related to dilator potency. Studies in isolated rat hearts showing P1Pi to significantly reduce BAY K 8644 induced coronary constriction, whilst having no effect on constrictions produced in response to a low sodium perfusate or caffeine point to a mechanism of action via inhibition of calcium entry through L-type calcium channels in smooth muscle. However , experiments using TEA and a high potassium perfusate suggest P1Pi may interact with coronary smooth muscle potassium channels. This contradicts the findings of Criddle et al. (1994) in the mesenteric vascular bed. In the myocardium, the ability of P1Pi to inhibit positive inotropic responses to noradrenaline, methoxamine and $PGF_{2\alpha}$ without affecting basal myocardial contractility or low sodium perfusate induced increases in contractility suggests an ability of P1Pi to interact with signal transduction mechanisms.

As the positive charge on the molecule is essential for coronary dilator activity, and charged molecules do not readily cross lipid bilayers, it is likely that P1Pi and related compounds are producing their effects on the sarcolemmal surface of the cell, to increase the transmembrane potential and hence reduce vascular and myocardial responsiveness. It is known that an increase in positive charge on the cell surface in the region of ion channels affects ion channel gating (Green & Andersen, 1991; Latorre et al., 1992). However, the interaction of P1Pi with currents through specific ion channels, eg. calcium or potassium channels, in myocardial and coronary smooth muscle cells requires further study, using electrophysiological techniques.

## Future Work

Future work would focus on the study of P1Pi in single cardic myocytes and smooth muscle cells, by fluorimetric measurement of intracellular calcium and electrophysiological techniques. In addition, the effects of P1Pi on both the coronary vascular and myocardial effects of ischaemia / reperfusion could be investigated further.

### 1. Fluorimetric Analysis.

The fluorimetric analysis studies would continue from those studies so far carried out in isolated cardiac myocytes, as well as using cultured vascular smooth muscle, to investigate further the effects of P1Pi on agonist-induced changes in intracellular calcium. Various agents which increase intracellular calcium via increased influx or release from the sarcoplasmic reticulum would be used. These studies could be performed in the presence and absence of extracellular calcium to investigate the relative effects of P1Pi on calcium entry or sarcoplasmic reticulum calcium release.

### 2. Electrophysiology.

The electrophysiological studies would focus on the interaction of P1Pi with L-type calcium channels in isolated myocytes using whole cell patch clamp techniques. This would establish whether P1Pi is able to modify the basal calcium current through L-type channels, as well as investigating the effect following activation with various agents eg. BAY K 8644, isoprenaline/noradrenaline, cholera toxin, forskolin and dibutyryl cyclic AMP. This will give a clearer picture as to whether P1Pi is inhibiting L-type calcium channel currents via a direct $G_S$ protein interaction, via inhibition of the activation of adenylyl cyclase or inhibition of the activation of protein kinase A. The effects of methoxamine and $PGF_{2\alpha}$ would also be

investigated to establish whether they are able to modify L-type calcium currents directly, and this will help to elucidate the mechanism for the inhibition of the positive inotropic actions of these agents by P1Pi in whole heart preparations. The effect of P1Pi on other currents, eg. T-type calcium channel currents, would also be investigated in myocytes.

The effect of P1Pi on calcium currents in vascular smooth muscle cells would also be investigated to establish whether P1Pi has a different mechanism of action in smooth muscle to that in cardiac muscle. The effect of P1Pi on other calcium channels in smooth muscle as well as L-type calcium channels, eg. R-type calcium channels, would be investigated. In addition, electrophysiological studies would be carried out to further investigate any interaction between P1Pi and the various $K^+$ channels present in myocardial and vascular smooth muscle.

**3. The effect of P1Pi on ischaemia / reperfusion.**

These studies would continue from those already carried out, ie. on 20 minutes ischaemia / reperfusion in the isolated rat heart. Firstly, to see whether higher concentrations of P1Pi can significantly inhibit the ischaemic contracture developed within the myocardium. Secondly, to establish the mechanism for the slowly developing coronary constriction produced on reperfusion, ie. whether the release of constrictor mediators is involved, in order to elucidate the mechanism for the P1Pi-mediated inhibition of constriction. Other experiments would investigate whether this inhibition can be mimicked by other vasodilators eg. calcium antagonists.

Additionally, the effect of P1Pi on more prolonged ischaemia could be studied; and also any protective effects of P1Pi against the development of arrhythmias following regional ischaemia / reperfusion.

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